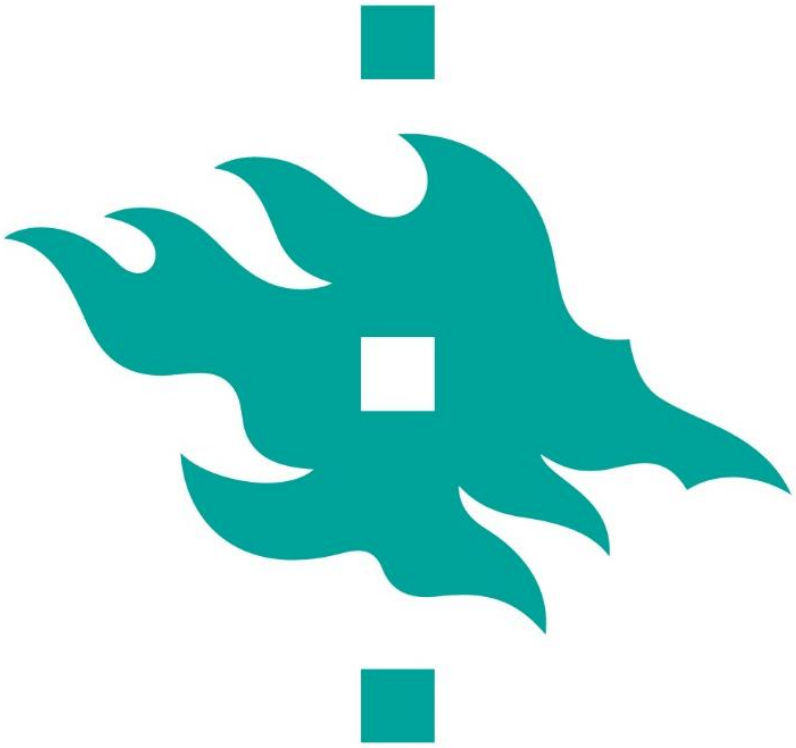

SH3 DOMAIN MEDIATED VIRUS-HOST CELL INTERACTIONS BY THE NONSTRUCTURAL PROTEIN 1 (NS1) OF INFLUENZA A VIRUS

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SH3 domain mediated virus-host cell interactions by the nonstructural protein 1 (NS1) of influenza A virus

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To my family

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LIST OF ORIGINAL PUBLICATIONS

I: Heikkinen LS, Kazlauskas A, Melén K, Wagner R, Ziegler T, Julkunen I, and Saksela K.
Avian and 1918 Spanish influenza A virus NS1 proteins bind to Crk/CrkL SH3 domains to activate host cell signaling.
J Biol Chem. 2008 Feb 29;283(9):5719-27.

II: Ylösmäki L, Schmotz C, Ylösmäki E, and Saksela K.
Reorganization of the host cell Crk(L)-PI3 kinase signaling complex by the influenza A virus NS1 protein.
Virology. 2015 Jun 19;484:146-152.

III: Ylösmäki L, Fagerlund R, Kuisma I, Julkunen I, Saksela K.
Nuclear translocation of Crk adaptor proteins by the influenza A virus NS1 protein.
Viruses. 2016 Apr 15;8(4).

ABSTRACT

Src homology 3 (SH3) domains are small modular protein structures that recognize and bind to short proline-rich sequence motifs in their ligand proteins. Viral proteins may also harbor such binding motifs and thereby serve as SH3 ligands in order to regulate the host cell signaling to support virus growth and replication, and to modulate virulence. The aim of this study was to examine if influenza A virus (IAV) might also use this strategy to take control of its host cells, and to characterize possible SH3 domain-containing host cell binding partners of IAV to establish their role in the cell biology of IAV infection.

IAVs cause seasonal epidemics and occasional pandemics that pose a major threat to human health. The nonstructural protein 1 (NS1) is an important virulence factor of IAV. It is a multifunctional protein that suppresses the host interferon response via multiple mechanisms. Another function of NS1 is to activate phosphatidylinositol-3 kinase (PI3K) signaling in the host cell through direct binding to the p85β regulatory subunit of PI3K. The NS1-induced activation of PI3K is required for efficient replication of many IAV strains.

We found that NS1 proteins from some IAV strains contain an SH3 binding site that mediates strong and selective binding to the N-terminal SH3 (nSH3) domain of Crk-family proteins, an important class of adaptor proteins involved in the coordination of cellular signal transduction. This Crk SH3 binding motif was present in the NS1 of infamous 1918 Spanish Flu pandemic virus as well as in many contemporary avian IAV strains. In contrast, it is not found in most NS1 proteins of seasonal human IAV strains. We found that the capacity of avian and Spanish Flu NS1 proteins to interact with Crk SH3 domains provided them with a greatly enhanced capacity to activate PI3K signaling. The molecular mechanism underlying this potentiation was found to be due to a reorganization of the natural PI3K-Crk complex by the SH3-binding competent NS1 protein. Of note, Crk proteins were found to indirectly (via p85β binding) contribute also to PI3K regulation by NS1 proteins of common human IAV strains that lack an SH3 binding motif and a capacity for direct Crk recruitment.

Moreover, we found that the role of the NS1/Crk interaction is not limited to PI3K regulation. We observed that binding of NS1 to the Crk SH3 domain induced a robust nuclear accumulation of the predominantly cytoplasmic Crk proteins. This nuclear translocation of Crk proteins was shown to lead to a change in tyrosine phosphorylation pattern of nuclear proteins.

In summary, our studies establish Crk adaptor proteins as important cellular co-factors exploited by the IAV virulence factor NS1 to manipulate host cell signaling. These results increase our understanding of the role of NS1 in IAV cell biology, and reveal possible new targets for future antiviral drug development aimed against critical host cell interactions rather than highly mutable viral proteins.

ABBREVIATIONS

ATP	adenosine triphosphate
AP-1	activator protein 1
BAD	Bcl2-antagonist of cell death
C-terminal	carboxyterminal
CARD	caspase recruiting domain
CPSF	cleavage and polyadenylation specificity factor
CPSF30	30 kDa subunit of the CPSF complex
CrkL	Crk-like
CRM1	chromosomal maintenance 1, exportin 1
cRNA	complementary RNA
cSH3	C-terminal SH3 domain
DNA-PK	DNA-dependent protein kinase
dsDNA	double-stranded DNA
EBV	Ebstein-barr virus
ED	effector domain
EGFR	epidermal growth factor receptor
eIF4GI	elongation and initiation factor 4GI
ER	endoplasmic reticulum
FAK	focal adhesion kinase
FasL	Fas-ligand
GST	gluthathione S-transferase
HA	hemagglutin
HCV	hepatitis C virus
HIV-1	human immunodeficiency virus-1
Hsp90	heat shock protein 90
IAV	influenza A virus
IFN	interferon
IRF3	interferon response factor 3
ISC	inter SH3 core
ISG	interferon stimulated gene
iSH2	inter SH2 domain
ISRE	interferon stimulated response element
JNK	c-jun N-terminal kinase
K_d	dissociation constant
LMP1	latent membrane protein 1
M1	matrix protein
M2	integral membrane protein, ion channel protein
MBP	maltose binding protein
MDA5	melanoma differentiation-associated gene 5
MEF	mouse embryonic fibroblast
MLK	mixed lineage kinase 3
MOI	multiplicity of infection
mRNA	messenger RNA
mTORC2	mammalian target of rapamycin complex 2
N-terminal	aminoterminal
NA	neuraminidase
NFκB	nuclear factor κB
NEP	nuclear export protein
NES	nuclear export signal

NLS	nuclear localization signal
NMR	nuclear magnetic resonance
NoLS	nucleolar localization signal
NP	nucleoprotein
NPC	nuclear pore complex
NS1	nonstructural protein 1
NS2	nonstructural protein 2
NS5A	nonstructural protein 5A
nSH3	N-terminal SH3 domain
OAS	2'-5'-oligo A synthetase
p110	catalytic subunit of PI3K
p85	regulatory subunit of PI3K
PA, PA-X	IAV polymerase subunits
PABP1	poly(A)-binding protein 1
PB1, PB1-F2	IAV polymerase subunits
PB2	IAV polymerase subunit
PBM	PDZ binding motif
PDK-1	phosphoinositide-dependent kinase-1
PEP	proline enriched phosphatase
PH	pleckstrin homology
PHLPP	PH domain and leucine-rich repeat protein phosphatase
PI3K	phosphatidylinositol-3-kinase
PIP_2	phosphatidylinositol (3,4)-bisphosphate
PIP_3	phosphatidylinositol (3,4,5)-trisphosphate
PKB	protein kinase B; Akt
PKR	protein kinase R
PPII	polyproline-2
PP2A	protein phosphatase 2A
pre-mRNA	precursor messenger RNA
PTEN	phosphatase of tensin homologue deleted on chromosome 10
RBD	RNA-binding domain
RIG-I	retinoic acid-inducible gene 1
RNA	ribonucleic acid
RNP	ribonucleoprotein
RTK	receptor tyrosine kinase
SH2	Src homology 2
SH3	Src homology 3
SKAP55	Src kinase-associated protein of 55 kDa
SOS	son of sevenless
ssRNA	single-stranded RNA
SUMO	small ubiquitin-like modifier
vtRNA	vaultRNA
vRNA	viral RNA
vRNP	viral ribonucleoprotein
WT	wild-type

1 REVIEW OF LITERATURE

1.1 Influenza A virus

Influenza A virus (IAV) belongs to the family *Orthomyxoviridae*, which is a family of enveloped viruses with segmented single-stranded RNA genome (Lamb and Krug, 2001). At the present, the family includes five other virus genera: *Influenzavirus B*, *Influenzavirus C*, *Thogotovirus*, *Quaranjavirus*, and *Isavirus* (ICTV, 2014). IAVs are further divided into different subtypes based on the antigenic variation of the surface glycoproteins, the hemagglutin (HA) and the neuraminidase (NA). Currently, there are 18 known subtybes for HA (H1-18), and 11 for NA (N1-11) (Fouchier et al., 2005; Lamb and Krug, 2001; Tong et al., 2012; Tong et al., 2013). The HA subtypes 1-16 and NA subtypes 1-9 have been isolated from the aquatic wild birds (CDC, 2015). In contrast, only a limited number of IAV subtypes have been isolated from humans. In addition to aquatic birds and humans, IAVs also infect a variety of animal species, including other birds, pigs, horses, seals, cats, and dogs (Wright and Webster, 2001). Recently, two new subtypes, H17N10, and H18N11 have been isolated form bats (Tong et al., 2012; Tong et al., 2013).

The aquatic birds of the orders *Anseriformes* (ducks, geese, swan, etc.) and *Charadriiformes* (gulls, terns, waders, etc.) are the major natural reservoir species for IAVs (Webster et al., 1992). In wild aquatic birds, the IAVs infect predominantly the epithelial cells of the lower intestinal tract. The transmission from bird to bird occurs via a fecal-oral route and the virus is maintained mainly as asymptomatic infections in the population. In contrast, domesticated birds of the order *Galliformes* (turkeys, chickens, quails, etc.) are susceptible for IAV infection after viral adaptation but they are not a reservoir host for the virus. The first mammalian IAV infection was clinically detected in swine during the Spanish Flu pandemic in 1918 (Taubenberger et al., 2001). Since that, IAVs have been isolated from numerous other mammalian host species as well, including humans, horses, dogs, and marine mammals. Swine are considered as a prime intermediate host for generation of novel IAVs which may have pandemic potential to humans, since they are susceptible for infections with both avian and human IAV strains (Webster et al., 1992).

The IAV genome is comprised of eight RNA segments. Depending on the virus strain, the eight segments encode up to 10-13 proteins (see Table 1) (Lamb and Krug, 2001). Two major surface glycoproteins HA and NA, the ion channel M2, and the matrix protein M1 make up the structure of the virion (see below). The NP (nucleoprotein) is bound to viral RNA (vRNA) to make up the viral ribonucleoproteins (vRNPs) to which the three viral polymerase subunits (PA, PB1 and PB2) are tightly associated. The nuclear export protein (NEP, formerly known as nonstructural protein 2, NS2) is also present in the virion, whilst nonstructural protein 1 (NS1), PB1-F2, PA-X, and N40 are expressed in the infected cells, but absent from the virion. Only the segments encoding NS1/NEP and M1/M2 are spliced to produce two proteins.

1.1.1 Disease and epidemics

In humans, IAV typically causes an acute infection of the upper respiratory tract. IAV infection is often characterized by the sudden onset of high fever, headache, muscle pain and severe malaise, together with a cough, sore throat, and running nose (Wright

and Webster, 2001). IAV causes seasonal epidemics among humans, which are estimated to result in about 3-5 million cases of severe illness, and about 250 000 - 500 000 deaths every year (WHO, 2014). Occasionally, when a novel subtype of IAV emerges, typically from an animal origin, global pandemics occur. In the 20th century, there have been four such pandemics. The most severe pandemic known of all time occurred in 1918-1919. The famous "Spanish Flu" has been estimated to have clinically affected approximately 500 million people, and caused the death of over 40 million people (Wang and Palese, 2013). Pandemics in 1957 ("Asian Flu") and 1968 ("Hongkong Flu") were less severe, yet having significant mortality rates. The most recent pandemic occurred in 2009, when an antigenically novel H1N1 virus emerged in swine and started to circulate in humans (Wang and Palese, 2013). This pandemic virus, called the "Swine Flu", is still circulating among humans.

Table 1. Influenza A virus genome segments, polypeptides and their functions. Adapted from Krug and Fodor, 2013.

Segment	Gene	Protein	Amino acids*	Protein mass (kDa)*	Protein function
1	PB2	PB2	759	84-87	Polymerase subunit, cap-binding
2	PB1	PB1	757	87-96	Polymerase subunit, nucleotide addition
		N40	718		Unknown
		PB1-F2	87		Apoptosis regulator/virulence factor
3	PA	PA	716	83-85	Polymerase subunit, endonuclease
		PA-X	252		Modulates host response/degrades host cel polymerase II transcripts
4	HA	HA	566	63	Surface glycoprotein, receptor binding
5	NP	NP	498	50-60	RNP component, viral replication
6	NA	NA	454	48-63	Surface glycoprotein
7	M	M1	252	25-28	Matrix protein
		M2	97	11-15	Ion channel
8	NS	NS1	230	25-27	Nonstructural protein, multifunctional
		NS2/NEP	121	12-14	Nuclear export protein

* approximation; there is variation between the strains

1.1.2 Structure of the virion

IAV virions can display different shapes, the most common one being close to spherical with a diameter of 80-120 nm (Fujiyoshi et al., 1994). The virions possess three subviral components: the envelope, the matrix layer, and the RNP core (see Figure 1 for schematic representation of the IAV virion). The viral envelope is a lipid bilayer derived from the host cell plasma membrane, containing both cholesterol-enriched lipid rafts and non-raft lipids (Lamb and Krug, 2001). Two transmembrane viral proteins, HA and NA, stick out from the lipid bilayer. The third transmembrane protein, the viral integral membrane protein (M2) is located within the lipid bilayer and serves as an ion channel. The viral matrix protein (M1) lies underneath the lipid bilayer, making up the matrix layer and linking the viral envelope to the viral RNP core. M1 interacts with the

cytoplasmic tails of HA, NA, and M2 on the outer side and the vRNA and NP on the inner side (Nayak et al., 2009; Rossman and Lamb, 2011). The IAV genome is segmented into eight negative-sense RNA strands, located inside the matrix shell. Each RNA strand is packed into ribonucleoprotein (RNP) complexes that consists of nucleoproteins (NP) and viral RNA polymerases (PA, PB1 and PB2).

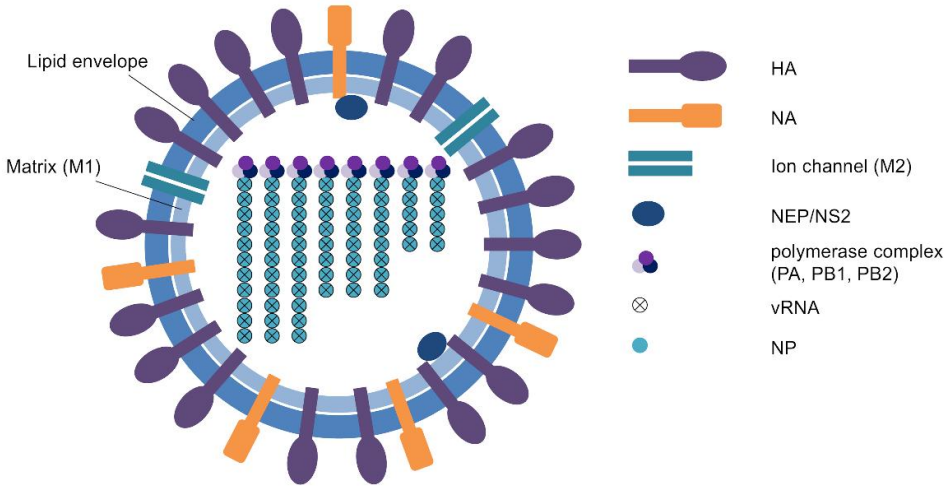

Figure 1. Schematic diagram of the structure of influenza A virus particle. Adapted from Lamb and Krug, 2001.

1.2 Influenza A virus life cycle

The IAV life cycle can be divided into five stages: 1) virus entry into the host cell; 2) entry of viral RNPs (vRNPs) into the nucleus; 3) transcription and replication of the viral genome; 4) export of the vRNPs from the nucleus; and 5) assembly and budding at the host cell membrane. In the following, the steps are discussed in more detail (see Figure 2 for schematic representation of IAV life cycle).

1.2.1 Entry

The HA of IAV is a homotrimer that binds to sialic acid containing cell surface receptors (Lamb and Krug, 2001). The specificity of the HA molecules in binding cell surface sialic acid receptors depends on the linkage between sialic acids and the carbohydrates they are bound. There are two of these linkages α(2,3) and α(2,6). IAVs isolated from humans recognize preferentially the α(2,6) linkage, whereas those from birds recognize the α(2,3) linkage. Pig trachea contain sialic acids linked to carbohydrates with both linkages and the swine has therefore been considered as a prime mixing vessel for the generation of IAV of pandemic potential to humans (Webster et al., 1992).

Upon binding to cell surface receptors, virus particles are endocytosed into early endosomes. About two thirds of the endocytosed IAV particles have been found to associate with clathrin-coated pits, and one third of the viruses are internalized via clathrin-independent pathway (Rust et al., 2004). Following the internalization, IAVs are transported to late endosomes where the acidic environment induces the fusion of the

viral and endosomal membrane, following the dissociation and degradation of M1 from RNPs and the release of viral genome.

1.2.2 Nuclear import of the viral genome

Unlike most other negative sense RNA viruses, the transcription and replication of IAV genome occurs in the nucleus of the infected cell (Herz et al., 1981, Jackson et al., 1982). Thus, in order for IAV to replicate, the vRNPs must enter the nucleus. Since they are too large for passive diffusion through nuclear pore complexes (NPCs), an active nuclear transport mechanism is required. The cytoplasmic vRNPs travel into the nucleus by using the importin-α - importin-β1 -dependent nuclear import pathway (Eisfeld et al., 2015). The transportation involves the recognition of nuclear localization signals (NLSs). All four vRNP associated proteins are reported to contain an NLS, but the NLS in NP seems to be sufficient for vRNP nuclear import (Cros et al., 2005).

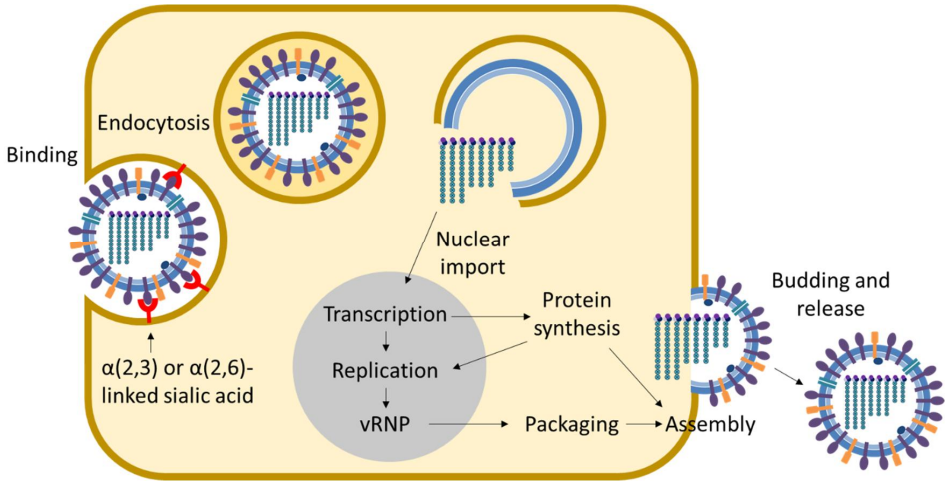

Figure 2. Schematic representation of influenza A virus life cycle. See the text for description. Adapted from (Shi et al., 2014).

1.2.3 Transcription and replication of the viral genome

After the nuclear import of vRNPs, they are transcribed to positive-sense RNA to produce viral mRNA. This is known as primary transcription. As the mature cellular RNAs, the viral mRNAs have poly(A) tails, and 5' caps. The 5'caps originate from cellular mRNA by process called "cap-snatching" (Bouloy et al., 1978; Plotch et al., 1979; Plotch et al., 1981). The viral polymerase PB2, which has endonuclease activity, binds to the 5' methylated caps of cellular mRNAs and steals them to be used as primer for primary transcription of vRNPs. The mechanism of viral mRNA polyadenylation is different from cellular mRNA polyadenylation. While cellular mRNAs are polyadenylated through cleavage at the polyadenylation signal by cleavage and polyadenylation specificity factor (CPSF) and subsequent addition of a poly(A) tail at the 3'end, the polyadenylation of viral mRNA is a result of the polymerase moving back and forth over a stretch of approximately 17 uridine residues located in the 5' end of the viral segment (Eisfeld et al., 2015). Only two of the eight viral mRNAs require splicing; segments 7 and 8 (Lamb

and Krug, 2001). The cellular splicing machinery is responsible for the splicing of the segments. The viral proteins are then translated in the cytoplasm by host cell ribosomes.

The replication of genomic vRNA happens in two steps: the initial synthesis of cRNA (the positive-sense RNA strand) that is complementary to the full-length vRNA; and the copying of cRNA into new negative-sense vRNAs (Lamb and Krug, 2001). The newly synthesized viral mRNA cannot act as a template for new vRNAs as it has a 5' cap derived from host and it is truncated relative to the full-length genomic segments. The viral polymerase initiates the cRNA synthesis in a cap-independent manner, and must be able to proceed through the polyadenylation site at the 5' end of vRNA. It is not well known, how the synthesis from viral mRNA is switched to the synthesis of cRNA. Soluble NP is required to prevent termination of the synthesis and polyadenylation at the poly(A) site (Beaton and Krug, 1984). Some host proteins have also been suggested to have a role in the process. For example, the direct interaction of viral polymerase with the large subunit of host cell Pol II enzyme (Engelhardt et al., 2005) has been proposed to improve the availability of capped RNA primers for viral transcription (Martinez-Alonso et al., 2016). In addition, all three viral polymerase subunits (PA, PB1 and PB2) are required for efficient synthesis of cRNA (Nakagawa et al., 1996). Finally, the cRNAs are used as a template to synthesize the negative-strand vRNAs. Soluble NP and polymerase subunits are added to the newly formed vRNAs to form vRNP complexes.

1.2.4 Export of the viral genome from the nucleus

IAV uses the cellular CRM1-dependent nuclear export pathway to actively mediate the transport of the newly synthesized vRNPs from the nucleus to the cytoplasm. There are two viral proteins essential for the vRNP nuclear export: M1 and NEP (Eisfeld et al., 2015). The M1 that is bound to the vRNP interacts with NEP which in turn interacts with the CRM1 via two NES (nuclear export signal) sequences, and contributes to the nuclear exit of vRNPs. Recent data implies that in addition to the interactions of NEP with M1 and CRM1, NEP also interacts with vRNP associated PB1 subunit of the polymerase complex (Brunotte et al., 2014) but further work is needed to clarify this process. In addition, the NP and some cellular protein kinases have been proposed to be involved in the nuclear export of vRNPs (Eisfeld et al., 2015).

Furthermore, there is growing evidence for apoptosis playing a critical role in the nuclear export of vRNPs. Wurzer et al. reported that inhibition of caspases resulted in reduced production of infectious virus progeny due to nuclear retention of vRNP complexes (Wurzer et al., 2003). This was specifically seen at a later stage of infection and was shown to be caspase 3-dependent. Recently, a study by Mühlbauer et al. showed that the induction of caspases during IAV infection increases the diffusion limit of nuclear pores allowing the vRNPs to passively diffuse out of the nucleus (Muhlbauer et al., 2015). These results suggest a model in which the vRNPs may exit the nucleus by two different methods depending on the time point of infection. In the intermediate steps of the virus life cycle, the vRNPs are exported by an active export mechanism which involves CRM1. At a later stage of infection, the caspase activity increases leading to the widening of nuclear pores, which allows the passive diffusion of vRNPs from the nucleus.

1.2.5 Virion assembly and budding

The newly synthesized viral proteins and vRNPs are transported to the lipid raft domains on the apical side of the cell plasma membrane where the virus assembly and budding occurs (Lamb and Krug, 2001). The transmembrane viral glycoproteins HA and NA as well as the ion channel M2 span the plasma membrane. On the cytoplasmic face of the membrane, M1 interacts with the cytoplasmic tails of HA, NA, and M2, and in turn interacts with the vRNPs.

Initiation of the virion budding is not well known. It has been suggested that the arrival of the vRNPs to the cell surface or their interaction with M1 promotes the budding (Lamb and Krug, 2001; Rossman and Lamb, 2011). Host cell protein UBR4, an ubiquitin ligase, has been shown to play an important role in the process (Tripathi et al., 2015). Binding of UBR4 with viral M2 ion channel promotes transport of viral proteins to the cell surface. The exact mechanism of virus budding is also unknown. NA has an important role in viral budding, by cleaving the sialic acid residues from the glycoproteins and from the surface of infected cells. This facilitates the efficient release of viral particles from the plasma membrane, and prevents self-aggregation of progeny virions. Other viral proteins, like HA, M1, and M2, have also been postulated to have different roles in viral budding but their contribution to the process is not clear.

1.3 Influenza A virus NS1 protein

NS1 is relatively small protein and it is the main viral antagonist of the innate immune response during IAV infection (Ayllon and Garcia-Sastre, 2015; Hale et al., 2008c). Despite its small size, NS1 has an outstanding number of described functions and interaction partners in the host cell. As the name “nonstuctural” implies, the NS1 protein is not a structural component of the IAV virion, instead it is expressed at very high levels in infected cells (Krug and Etkind, 1973).

1.3.1 Stucture of NS1

The shortest vRNA segment (segment 8) is transcribed into two mRNAs, which encode the nonstructural protein 1 (NS1) and nuclear export protein (NEP) (Inglis et al., 1979; Lamb and Choppin, 1979; Lamb and Krug, 2001). NS1 and NEP share 9 N-terminal amino acid residues, as the NS1 protein is translated from the unspliced primary mRNA transcript, whereas NEP is translated from a spliced transcript (Lamb and Lai, 1980). The splicing procedure is regulated, and only ~10-15 % of the segment 8 is spliced to encode NEP (Robb et al., 2010).

NS1 is a small, approximately 26 kDa, protein that has a strain specific length of 230-237 amino acids (see Figure 3 for schematic representation of NS1 protein) (Lamb and Krug, 2001). NS1 consists of two distinct globular domains: an aminoterminal (N-terminal) RNA-binding domain (RBD, amino acids 1-73), and a carboxyterminal (C-terminal) effector domain (ED, amino acids 85-230/7) separated by a short linker region (Ayllon and Garcia-Sastre, 2015). The two domains are well conserved within strains but the flexible linker region and disordered C-terminal tail display significant sequence variability. The strain specific length of the NS1 depends on amino acid deletions and insertions in these regions. For example, the linker region of highly pathogenic avian H5N1 IAV strains isolated after 2000 carry a deletion of five amino acids in the linker region and the NS1 of human IAVs isolated between 1950 and 1989 has a seven amino

acid extension in the C-terminal tail (Dundon and Capua, 2009; Melen et al., 2007). Two RBD associates to form homodimers with a six-helix antiparallel bundle (Cheng et al., 2009; Liu et al., 1997). Dimerization is essential for its binding to double-stranded RNA (dsRNA) (Chien et al., 1997; Qian et al., 1995; Wang et al., 1999). The arginine residue at position 38 is absolutely required (Wang et al., 1999) but other adjacent amino acids also participate in dsRNA binding (Cheng et al., 2009). The ED, which is formed by seven β-strands and three α-helices, also dimerizes (Aramini et al., 2011; Hale et al., 2008a). The interaction between EDs takes place through the highly conserved tryptophan residues at position 187 (Kerry et al., 2011). ED binds to numerous cellular proteins and mediates many NS1 functions (see Figure 3) (Ayllon and Garcia-Sastre, 2015; Hale et al., 2008c).

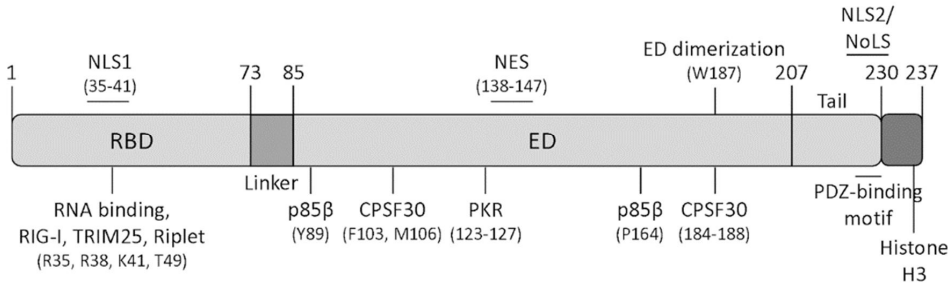

Figure 3. Schematic representation of the primary structure of NS1 protein, together with its known interaction partners. See the text for description and abbreviations. Adapted from Ayllon and Garcia-Sastre, 2015.

1.3.2 Intracellular localization of NS1

NS1 protein has distinct roles in the nucleus and the cytoplasm. For example, CPSF30 binding takes place in the nucleus, while regulation of RIG-I pathway happens in cytoplasm. Thus, the regulation of NS1 localization is important. Soon after virus infection, newly synthesized NS1 accumulates in the nucleus, but is transported to the cytoplasm at late time points of infection. The localization of NS1 is mediated by one or two nuclear localization signals (NLS) as well as by one nuclear export signal (NES) (Forbes et al., 2013; Han et al., 2010; Melen et al., 2007). NLS1 is found between amino acids 35 and 41, overlapping with the dsRNA binding sequence in RBD (Greenspan et al., 1988; Melen et al., 2007). It is well-conserved among IAV strains, while C-terminally located NLS2 is virus strain-specific (Greenspan et al., 1988; Melen et al., 2007; Melen et al., 2012). In addition, a virus strain specific nucleolar localization signal (NoLS) has been identified. NoLs overlaps with NLS2, and it requires additional basic residues at positions 224 and 229 (Melen et al., 2007; Melen et al., 2012). The nuclear export of NS1 is regulated by NES, which is located between amino acids 138-147, leucine residues 144 and 146 being critical for its function (Li et al., 1998).

1.3.3 Post-translational modifications of NS1

NS1 has been found to be modified in three ways: phosphorylation, ISG15 (interferon-stimulated gene 15) modification, and SUMO (small ubiquitin-like modifier) modification. These modifications have been reported to affect the function of NS1 and viral replication.

NS1 is phosphorylated at residues S42, S48, T49, and T215 (Hale et al., 2009; Hsiang et al., 2012; Kathum et al., 2015). The substitution of T215 to alanine in A/Udorn NS1 resulted in attenuation of viral replication (Hale et al., 2009). This was shown later not to be because of phosphorylation of this site, but because of a deleterious structural change of NS1 due to the mutation (Hsiang et al., 2012). Out of the other phosphorylated residues, only the phosphorylation of S42 and T49 was shown to have a relevant role (Hsiang et al., 2012; Kathum et al., 2015). The substitution of residue 42 in A/Udorn strain affected dsRNA binding and consequently replication, while the phosphorylation of T49 was shown to inhibit the association of NS1 with dsRNA, RIG-I and TRIM25.

NS1 has been shown to be conjugated to two ubiquitin-like proteins ISG15, an interferon stimulated ubiquitin homologue (Tang et al., 2010; Zhao et al., 2010) and SUMO (Pal et al., 2010). Lysine residue 41 (K41) in NS1 RBD was reported to be the main target for ISGylation by ISG15, and this modification resulted in disruption of interaction with importin α, but had no effect on the RNA binding activity, which is also mediated by K41 residue (Zhao et al., 2010). Two lysine residues in the C-terminal region of NS1, K219 and K221, have been identified as the sites for sumoylation (Xu et al., 2011). The sumoylation of these sites seems to enhance the stability of the NS1 protein, and substitution of K to E in these sites in A/WSN strain resulted in slower replication of the virus.

1.3.4 Inhibition of interferon production by NS1

Innate interferon (IFN) response is one of the first barriers a virus faces when infecting a host cell. It serves as a potent antiviral mechanism that limits the replication and spread of viruses (Randall and Goodbourn, 2008). Interferons are soluble cytokines and the expression of IFN-α, IFN-β, IFN-λ, and IFN-γ is induced upon viral infection. They act in both autocrine and paracrine manner to upregulate interferon stimulated genes (ISGs). ISGs are involved in development of an antiviral state in host cell and in alerting neighboring cells of the incoming thread. Some ISGs may also be induced directly by a viral infection. The innate IFN response also activates the specific adaptive immune response. Pathogens have developed a number of different strategies to overcome, limit, or hide from the IFN system and establish successful infection.

One of the most important functions of IAV NS1 protein is its role in the inhibition of IFN production. Studies with recombinant IAVs that do not express NS1 or that express truncated forms of NS1, have revealed the essential role for NS1 to counteract the host IFN response (Egorov et al., 1998; Fernandez-Sesma et al., 2006; Garcia-Sastre et al., 1998). The IAV strain A/PR8/8/34 (A/PR8) with NS1 deletion replicated to titres ~1000-fold lower than wt A/PR8 virus in IFN-competent cells (Garcia-Sastre et al., 1998). By contrast, in IFN-deficient cells, Vero cells, virus lacking the NS1 replicated almost as effective as the wt virus. Egorov et al. generated IAVs with truncated forms of NS1 with varying lengths (Egorov et al., 1998). They reported that while the replication of a virus with NS1 coding the first 124 amino acids was only slightly affected, the production of viruses with shorter forms of NS1 were markedly lowered in IFN-competent systems. Mutations of arginine 38 and lysine 41 greatly impaired the ability of NS1 to block interferon production (Pichlmair et al., 2006). The length of NS1 did not influence virus replication in IFN-incompetent cells. In addition, cells infected with IAV lacking the NS1

produce more IFN-α and IFN-β than wt virus (Fernandez-Sesma et al., 2006). Together, these studies indicated, and further studies have proved, that NS1 is an important factor limiting the host innate IFN response during IAV infection. NS1 has been shown to inhibit host cell interferon production both pre- and post-transcriptionally and different IAV strains have evolved diverse strategies to counteract host innate IFN-mediated antiviral response with NS1 protein (Hayman et al., 2007; Kuo et al., 2010).

1.3.4.1 Inhibition of the RIG-I pathway

RIG-I (retinoic acid-inducible gene 1) is a cytoplasmic RNA helicase that detects viral genomic single-stranded RNA (ssRNA) bearing 5' phosphates upon IAV infection, which leads to the activation of RIG-I pathway resulting in the induction of the synthesis of type I IFNs (Schlee and Hartmann, 2010). The binding of vRNA to RIG-I triggers a conformational change, exposing two N-terminal caspase recruiting domains (CARDs) (Myong et al., 2009). The second CARD of RIG-I is then ubiquitinated by the E3 ligases TRIM25 and RIPLET (Gack et al., 2009; Oshiumi et al., 2009). The activation of RIG-I pathway leads to the phosphorylation and nuclear translocation of transcription factors, such as IRF3 (interferon response factor 3), AP-1 (activator protein 1) and NFκB (nuclear factor κB), which drive the transcription of the type I IFN genes (McWhirter et al., 2005).

The NS1 protein of IAV has been shown to inhibit the activation of IRF3 (Talon et al., 2000), NFκB (Wang et al., 2000) and AP-1 (Ludwig et al., 2002). Recently, it has been found that this inhibition takes place at the RIG-I pathway and NS1 has different ways to limit the activation to repress the IFN expression. NS1 may inhibit RIG-I signaling by forming a complex with RIG-I itself (Jureka et al., 2015; Pichlmair et al., 2006) or by interacting with the ubiquitin ligases TRIM25 (Gack et al., 2009) and RIPLET (Rajsbaum et al., 2012) that are responsible for activation of the RIG-I pathway. Interaction of NS1 with TRIM25 blocks the E3 ligase activity on the CARD domains of RIG-I, and mutations in TRIM25 interaction site in NS1 cause viral attenuation and higher IFN induction (Gack et al., 2009). The association of NS1 with RIPLET also prevents the activation of RIG-I (Rajsbaum et al., 2012). The interaction site responsible for TRIM25 and RIPLET interaction seems to be different, and the interaction properties to these proteins by NS1 are virus strain specific (Rajsbaum et al., 2012). In addition, the direct interaction between NS1 and RIG-I has so far been reported only for Spanish Flu NS1, whereas the NS1 from IAV strain A/Udorn/73 (A/Udorn) was unable to interact directly with RIG-I (Jureka et al., 2015). Thus, the mechanism of RIG-I inhibition seems to have evolved individually in NS1 proteins of different IAV strains.

The RNA-binding property of NS1 has also been postulated to be one mechanism for NS1 to limit the activation of RIG-I pathway by sequestering vRNAs away from RIG-I. However, the affinity of NS1 to RNA is much lower than that of RIG-I (Chien et al., 2004). On the other hand, mutations of the important residues for NS1 RNA binding site (arginine R38 and lysine K41) greatly impair the ability of NS1 to block interferon production (Pichlmair et al., 2006). In addition, mutations at these residues also abrogate the interactions of NS1 with RIG-I, TRIM25 and RIPLET (Gack et al., 2009; Pichlmair et al., 2006; Rajsbaum et al., 2012). Recently, another amino acid in close proximity to the RNA-binding site on NS1 has been added to the RIG-I story: threonine 49 (T49) (Kathum et al., 2015). This residue was shown to impair the interaction of NS1 with dsRNA, RIG-I and TRIM25 when phosphorylated. The phosphorylation of T49

happens at late time points of infection, and was suggested to act as a spatial-temporal regulator of NS1 to direct its function from the inhibition of innate immunity to its other functions to facilitate IAV infection (Kathum et al., 2015).

1.3.4.2 Inhibition of host gene expression

The IAV NS1 protein inhibits the production of IFNs and other antiviral proteins also by suppressing the host gene expression. The CPSF (Cleavage and Polyadenylation Specificity Factor) catalyzes the addition of poly(A) tails to cellular pre-mRNAs (Colgan and Manley, 1997; Wahle and Keller, 1996). NS1 binds to CPSF30, the 30 kDa subunit of the CPSF complex, and inhibits the 3' end processing of cellular pre-mRNAs (Nemeroff et al., 1998). The unprocessed pre-mRNAs accumulate in the nucleus, and cellular mRNA processing in the cytoplasm is inhibited (Das et al., 2008). Thus, the translation of IFNs and other antiviral genes is blocked. Since the polyadenylation of viral mRNAs is catalyzed by the viral polymerase, the viral transcripts are not affected by the repression of CPSF (Robertson et al., 1981). The sequestered pre-mRNAs in the nucleus have also been suggested to provide a steady pool of capped 5' ends for cap snatching by the viral polymerase (Nemeroff et al., 1998).

There are several NS1 residues that have been reported to be relevant for the CPSF30 interaction. The interaction is mediated by the conserved hydrophobic residues 184-188, of which the W187 residue is also involved in NS1 ED dimerization (Das et al., 2008). Indeed, the dimerization of NS1 is important for the CPSF30 interaction. The interaction is further stabilized by two NS1 amino acids, phenylalanine F103 and methionine M106 (Kainov et al., 2011; Kochs et al., 2007; Twu et al., 2006). Mutation of these two amino acids does not abrogate the interaction, only reduces the binding of NS1 to CPSF30. The capacity to bind CPSF30 and to suppress gene expression varies between different IAV strains. Most of the avian and all human isolate IAV strains reported from 1998 onward share a strong binding capacity to CPSF30 (Ayllon and Garcia-Sastre, 2015). In contrast, the A/PR8 strain that is widely used in laboratories, and the highly pathogenic H5N1 avian IAV strain involved in the 1997 human outbreak in Hong Kong lack this property due to amino acid residue substitution at positions 103 and 106 (Kochs et al., 2007; Twu et al., 2006). NS1 of 2009 pandemic (The Swine Flu) IAV is also defective in binding to CPSF30 due to amino acid substitutions at positions 108, 125, and 189 (Hale et al., 2010c).

In contrast to CPSF30-inhibition, which un-specifically blocks the expression of all host genes, some IAV strains have another, specific mechanism for the inhibition of host gene expression. NS1 proteins of H3N2 IAVs isolated since 1989 have a 7 amino acid extension in their C-terminus (being 237 amino acids long) (Ayllon and Garcia-Sastre, 2015). The C-terminus of these NS1 proteins contains a sequence, ARSKV, which is very similar to the motif ARTKQ found on histone H3. The tail of NS1 acts as a mimic for H3 and binds to PAF1 transcription-elongation complex which regulates genes involved in the IFN response (Marazzi et al., 2012). Consequently, the elongation of host gene transcription is blocked.

1.3.4.3 JNK pathway

IAV infection has been reported to trigger the phosphorylation and activation of c-jun N-terminal kinase (JNK) pathway (Ludwig et al., 2001). JNK phosphorylation leads to

activation of AP-1 transcription factors ATF-2 and c-Jun, which are required for the induction of IFN-β production. IAV has been reported to activate JNK by two means: directly by NS1 protein or through vRNA recognition by RIG-I (Nacken et al., 2014). The activation of JNK seems to have a virus-supportive function, since the chemical inhibition of JNK resulted in decreased virus replication (Nacken et al., 2012). The ability of NS1 to activate JNK depends on subtype specific sequence variation at amino acid 103 (Nacken et al., 2014). Phenylalanine at this position, the same amino acid involved in stabilization of CPSF30 interaction, was shown to be important for JNK activation. On the other hand, NS1 protein of some IAV strains have been reported to inhibit JNK activation (see below) (Hrincius et al., 2010; Ludwig et al., 2002). Partly it may be indirect, due to the ability of NS1 to inhibit RIG-I.

1.3.4.4 Inhibition of the activity of antiviral proteins PKR and OAS

NS1 limits also interferon response at the post-transcriptional level by inhibiting two ISG products, PKR (protein kinase R) and OAS (2'-5'-oligo A synthetase). The transcription of PKR and OAS is upregulated upon virus infection, they play a key role in host cell antiviral response and they are activated by binding to dsRNA (Ayllon and Garcia-Sastre, 2015; Krug, 2015). For PKR, studies with influenza B virus have shown that cytoplasmic vRNP can function as an activator (Dauber et al., 2009) which is also believed to be true in IAV infected cells (Dauber and Wolff, 2009). The recognition of dsRNA or vRNPs leads to autophosphorylation and activation of PKR. The activated PKR then binds and phosphorylates its best studied target, the eukaryotic translation initiation factor 2 (eIF2α), ultimately leading to inhibition of translation initiation. The PKR mediated block of translation impairs viral spread, since the replication of viruses critically depends on the cellular translation machinery.

OAS catalyzes the formation of 2'-5'-poly(A) chains from ATP to activate the otherwise latent RNase L, which repress viral infection by degrading single-stranded RNA (Silverman, 2007). OAS/RNaseL may also enhance the activation of IFN transcription, since the degradation products may bind to and activate RIG-I. Also, the PKR has been reported to play an additional role in IFN-induction and the host apoptotic responses (Garcia et al., 2006; Silverman, 2007).

For long time, it was believed that NS1 inhibits PKR activation by competing for binding to dsRNA (Hatada et al., 1999; Lu et al., 1995). However, the activity of PKR is efficiently limited also by RNA-binding defective NS1 (Li et al., 2006; Min and Krug, 2006). In addition, the affinity of NS1 to dsRNA is much lower compared to PKR, so the competition is out ruled (Li et al., 2006). Instead, it has been shown that NS1 prevents the activation of PKR by direct interaction, and the amino acids 123-127 in NS1 have been reported to be important (Min et al., 2007). A recent study by Li et al. has provided a new insight to the regulation of PKR by NS1 protein (Li et al., 2015). NS1 triggers the upregulation of vtRNAs (vaultRNA), which are part of a large ribonucleoprotein particles located in the cytoplasm. This results in inhibition of PKR activity. In contrast, the dsRNA binding affinity of OAS is low enough for NS1 to outcompete it (Min and Krug, 2006). Thus, the mechanism for NS1 to inhibit OAS/RNaseL may be by sequestering the dsRNA away from OAS. Recently, RNA helicase MDA5 (melanoma differentiation-associated gene 5), a relative of RIG-I, was shown to be involved in the enhancement of the antiviral

response through the recognition of the OAS/RNaseL system (Benitez et al., 2015) and PKR (Pham et al., 2016).

1.3.5 NS1 in apoptotic pathways

Apoptosis is considered to be a cellular antiviral mechanism to limit viral replication. As such, IAVs have developed various means by which to regulate this host defense strategy and NS1 is reported to have both pro- and anti-apoptotic functions (Hale et al., 2008c). The anti-apoptotic functions of NS1 can be linked to its ability to suppress IFN production (Zhirnov et al., 2002). While A/PR8 IAV with deleted NS1 induced higher levels of apoptosis than wt A/PR8 virus in IFN-competent system, similar levels of apoptosis was observed in IFN-incompetent system with both viruses. This was speculated to indicate that the anti-apoptotic function of NS1 is IFN-dependent.

As PKR and OAS/RNase L have also been reported to play a role in apoptosis, the inhibition of these proteins by NS1 may lead to suppression of cell death (Min and Krug, 2006; Takizawa et al., 1996). NS1 also inhibits the JNK-pathway, pathway that is linked to activation of apoptosis (Hrincius et al., 2010; Ludwig et al., 2002). As well, the association of NS1 to Scribble through PDZ-domain binding has been reported to be anti-apoptotic (see below) (Liu et al., 2010). In addition, activation of the host cell PI3-kinase (PI3K) pathway (see below) has been described as an additional, IFN-independent method by which NS1 may limit the induction of apoptosis (Ehrhardt et al., 2007; Shin et al., 2007b; Zhirnov and Klenk, 2007). However, NS1 has also been reported to have pro-apoptotic functions. Transient expression of NS1 from several human, swine and avian IAV isolates have been reported to induce apoptosis in cultured cells (Han et al., 2012; Lam et al., 2008; Lam et al., 2011; Schultz-Cherry et al., 2001; Zhang et al., 2011). The mechanism how NS1 induces apoptosis is not known. Zhang et al. (2011) proposed, that NS1 interacts with Hsp90 (heat shock protein 90), which would promote the activation of caspase cascade (Zhang et al., 2011).

It may be that controversial apoptosis mechanisms reported by NS1 are caused by the differences in virus strain NS1 proteins, as well as the host systems used in the studies. It may also be that the differential apoptosis control is temporally regulated, so that apoptosis is inhibited early during infection (promotes the replication of the genome), whilst enhanced at late time points of infection (increases the release of progeny virions).

1.3.6 Interactions of NS1 with other host cell factors

1.3.6.1 PDZ domain mediated interactions

The PDZ domains bind to a specific PDZ binding motif (PBM) that is typically found at the extreme C-terminus of a target protein, although in some cases the binding motif can have an internal location (Javier and Rice, 2011). The IAV NS1 protein displays PBM at the very C-terminus of the NS1 sequence (Obenauer et al., 2006). In avian IAV NS1 protein the consensus sequence for PBM is either ESEV or EPEV, whereas in human IAV isolates the consensus sequence is RSKV. Only the NS1 proteins from avian IAV isolates have so far been reported to bind to PDZ domain containing proteins, which have anti-apoptotic or cell polarity regulatory functions (Javier and Rice, 2011). The interaction of NS1 PBM with two PDZ domains of Scribble protein protected the IAV infected cells from apoptosis (Liu et al., 2010). Furthermore, the interaction of NS1 with PDZ domains of

Scribble and Dlg-1 was reported to disrupt cellular tight junctions to increase the permeability of infected cells (Golebiewski et al., 2011). NS1 PBM also mediates the binding and inactivation of MAG-1, which leads to higher IFN-β production (Kumar et al., 2012). This rather unexpected function for NS1 seems to be masked by other IAV anti-IFN functions and the relevance of this interaction to IAV infection remains to be solved.

The ESEV-sequence was found to increase the replication and virulence of human A/WSN (A/WSN/33) IAV strain (Jackson et al., 2008) and H3N2 IAV strains (Liu et al., 2010) when introduced to the NS1 sequence of these viruses. In addition the avian H7N1 strain was shown to benefit from the ESEV-sequence since the mutation of the motif resulted in lower replication of the virus (Soubies et al., 2010). In contrast, when the ESEV-sequence was introduced into highly pathogenic H5N1 virus it did not affect virulence in a mouse model (Zielecki et al., 2010). Similarly, the deletion of PBM from NS1 in low-pathogenic avian H7N1 virus strains had no effect on replication (Soubies et al., 2013). Likewise, the restoration of PBM in the NS1 of the 2009 H1N1 pandemic virus did not have an effect on the viral growth in cultured cells (Hale et al., 2010b) but had a positive effect on viral transmission in a guinea pig model (Kim et al., 2014). Thus, the requirement of PBM for viral replication is strictly virus strain specific.

1.3.6.2 SH3 domain mediated interactions

The Src homology 3 (SH3) domains bind to proline-rich sequences to transmit various signals (see below). As first reported in study **I** of this thesis, the Spanish Flu and many avian IAV NS1 proteins contain a functional SH3 domain binding motif close to their C-terminus. This motif mediates the binding of NS1 to the SH3 domains of Crk adaptor proteins. The NS1 proteins of human IAVs do not possess this motif. Hrincius et al. showed that downregulation of Crk proteins resulted in impaired propagation of IAVs that contain SH3 binding competent NS1 protein due to the inhibition of IAV-mediated activation of c-jun N-terminal kinase (JNK) signaling pathway (Hrincius et al., 2010). Especially, CrkI was shown to be important for the inhibition of JNK-pathway and subsequent inhibition of apoptosis. In another study by the same group, NS1 was demonstrated to reduce the activity of tyrosine kinase c-Abl (Hrincius et al., 2014). The inhibition was shown to be mediated by the Crk-binding capacity of NS1 but also by a direct interaction of NS1 with c-Abl. The reduced activity of c-Abl led to lower basal levels of Crk phosphorylation and severe cytopathic effects of infected cells. Chemical inhibition of c-Abl impaired propagation and pathogenicity of avian IAV. The NS1-mediated inhibition of c-Abl was further shown to result in severe lung injury and to facilitate secondary bacterial infections in mice when functional SH3 binding motif was introduced into the NS1 protein of human IAV strain A/PR8 (Hrincius et al., 2015).

1.3.7 Regulation of viral RNA and protein synthesis by NS1

The gene expression of IAV can be divided into an early and late phase. NS and NP vRNAs are replicated and transcribed in the early phase, while in the late phase all 8 vRNAs are replicated and transcribed (Shapiro et al., 1987; Skehel, 1973). Several studies have provided evidence that NS1 is involved in the regulation of vRNA synthesis. Mutation in the NS1 sequence at residues 123 and 124 resulted in deregulation of the normal time course of vRNA synthesis, with both early and late vRNAs being replicated and transcribed at high levels already at very early times after infection (Min et al., 2007), while deletion of whole ED impaired the transcription of late genes (Maamary et al.,

2012). The mechanism how NS1 regulates temporally vRNA synthesis is not known. It has been suggested that the interaction of NS1 with virus polymerase complex plays a role in the process (Kuo and Krug, 2009). In addition, a recent study by Chen et al. suggested that the inhibition of DDX21, a helicase able to block the viral polymerase complex, by NS1 may be the key factor for this regulation (Chen et al., 2014).

In IAV infected cells, the translation of host cell mRNAs is strongly inhibited, so the viral proteins may be translated efficiently (Garfinkel and Katze, 1993). NS1 has also shown to stimulate the synthesis of viral proteins (de la Luna et al., 1995). Amino acids at positions 25/26, 48, and 67 in the RBD of NS1 have been reported to be essential for the stimulation of translation (Kainov et al., 2011). The NS1 binds to 5' untranslated region of viral mRNA (Park and Katze, 1995) and to several proteins involved in eukaryotic translation, such as eIF4GI (elongation and initiation factor 4GI) (Aragon et al., 2000) and PABP1 (Burgui et al., 2003), as well as hStaufen (Falcon et al., 1999), a dsRNA binding protein involved in the transport of mRNA to activate translation sites. The proposed model is that NS1 recruits these translation factors to the 5' ends of viral mRNAs to favor the translation of viral proteins.

1.4 PI3K/Akt pathway

1.4.1 An overview of the PI3K/Akt pathway

The phosphatidylinositol-3-kinases (PI3Ks) are members of a conserved family of intracellular kinases that exhibit both protein kinase and lipid kinase activity. They are classified in three main classes (I-III) according to their substrate specificity and sequence homology. Class I PI3Ks are further divided into two subclasses: IA and IB. Class IA PI3Ks are heterodimers that consist of a regulatory subunit and a catalytic subunit. In mammalians there are three class IA catalytic (p110 α, β and δ) and five regulatory (p85α, p85β, p55γ, p55α, and p50α) subunits known (Engelman et al., 2006). Cellular responses that involve PI3K signaling include survival, proliferation, trafficking, and regulation of immune function. Basal activity of PI3K/Akt signaling ensures cell survival, while inactivation of the pathway results in apoptosis.

The activation of PI3K is tightly controlled (see Figure 4 for schematic representation of activation of PI3K/Akt signaling). The PI3K pathway is activated upon ligand binding on cell surface receptor tyrosine kinases (RTKs) which leads to dimerization and autophosphorylation of RTKs. The regulatory subunit p85 contains an SH2 (Src-homology 2) domain which binds to tyrosine phosphorylated YXXM motifs on the RTKs. This triggers the activation of p110 catalytic subunit leading to conversion of phosphatidylinositol (3,4)-bisphosphate (PIP_2) lipids to phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). PIP_3 serves as a second messenger interacting with PH (pleckstrin homology) domain-containing proteins. PI3K has several downstream signaling mediators. One important mediator is Akt (also known as PKB; protein kinase B) that binds to PIP_3, allowing PDK-1 (phosphoinositide-dependent kinase-1) to phosphorylate threonine at the position 308 (T308) on Akt. The full activation of Akt requires another phosphorylation of serine at position 473 (S473). The kinase responsible for S473 phosphorylation is either mTORC2 (mammalian target of rapamycin complex 2) (Sarbassov et al., 2005) or DNA-PK (DNA-dependent protein kinase) (Feng et al., 2004) depending on the initial stimulus.

Akt is a serine/threonine kinase which upon full activation phosphorylates many target proteins (Engelman et al., 2006). Regulation of apoptosis is an important function ascribed to PI3K/Akt -signaling. Akt promotes cell survival by phosphorylating and inactivating pro-apoptotic proteins, such as FasL (Fas-ligand), BAD (Bcl2-antagonist of cell death) and caspase-9. Active Akt is also involved in other cellular functions, including angiogenesis, metabolism, growth, protein synthesis, proliferation, and survival.

PI3K/Akt signaling is negatively regulated by different phosphatases. Dephosphorylation of Akt is mediated by phosphatases PP2A (protein phosphatase 2A) (Millward et al., 1999), and PHLPP (PH domain and leucine-rich repeat protein phosphatase) (Gao et al., 2005). The most important negative regulator of PI3K signaling is the PTEN (phosphatase of tensin homologue deleted on chromosome 10) which dephosphorylates PIP_3 back to PIP_2 (Lam et al., 2008; Lam et al., 2011; Stambolic et al., 1998).

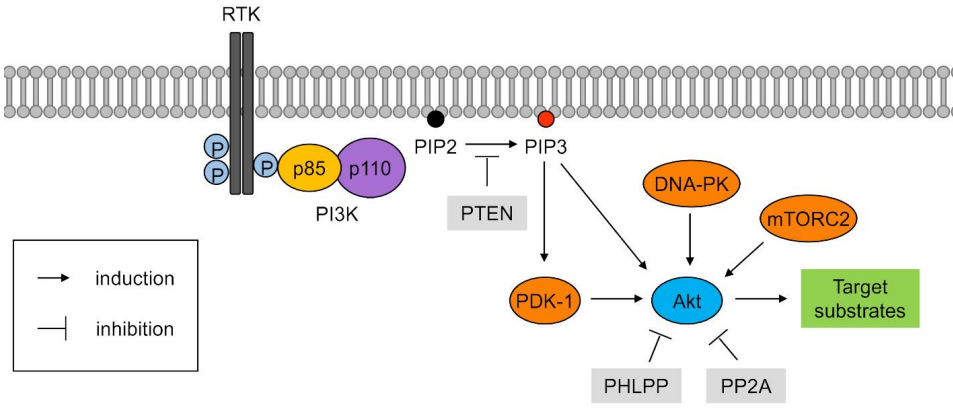

Figure 4. Schematic representation of PI3K/Akt pathway activation. See the text for description. After Engelman et al., 2006; Hemmings and Restuccia, 2015.

1.4.2 Targeting of the PI3K/Akt pathway by viruses

Since the host cell PI3K/Akt signaling pathway has a critical regulatory role in many cellular processes, including survival, proliferation, RNA processing, translation, and regulation of immune function, viruses have evolved widely varying ways to target it (Diehl and Schaal, 2013; Engelman et al., 2006). Many enveloped viruses activate the PI3K/Akt pathway to facilitate the endocytic uptake of virions. For example, the activation of PI3K during endocytic uptake of Ebola viruses is required since the inhibition of the pathway leads to suppression of virus infection at an early step (Saeed et al., 2008). Vaccinia virus, herpes simplex virus type-1 and IAV also requires PI3K activation for host cell uptake (Diehl and Schaal, 2013; Ehrhardt et al., 2006).

In addition to entry, many viruses manipulate PI3K/Akt signaling to control apoptosis during the viral infection (Diehl and Schaal, 2013). The viral protein LMP1 (latent membrane protein 1) of Epstein-Barr (EBV) virus activates the PI3K/Akt pathway by direct interaction with the p85 subunit, resulting in cell survival (Dawson et al., 2003). Rotavirus NSP1 and hepatitis C virus (HCV) NS5A (nonstructural protein 5A) also interact with p85 to induce PI3K/Akt pathway (Bagchi et al., 2010; Street et al., 2004). NS5A binds

to p85 and the interaction is mediated by the SH3 domain of p85. In addition, herpes simplex virus-1, respiratory syncytial virus, coxsackie virus B3, rubella virus, HIV-1, and IAV prevents apoptosis by inducing the PI3K/Akt pathway during infection (Diehl and Schaal, 2013). Some viruses utilize the PI3K/Akt pathway to regulate the translation of viral proteins by activating mTOR (mammalian target of rapamycin), a downstream target of Akt. EBV LMP2A, adenovirus E4-ORF1, and E6 and E7 proteins of human papilloma virus are examples of viral proteins which have been reported to activate mTOR during infection (Diehl and Schaal, 2013).

1.4.3 Induction of the PI3K/Akt pathway by NS1 protein

The PI3K signaling is activated twice in IAV infected cells (Ehrhardt et al., 2006). The attachment and entry of the virus cause the first, a transient PI3K activation, which is related to the uptake of virus by endocytosis. The second wave of activation, sustained activation, appears 4-6 hours post-infection (Ehrhardt et al., 2006; Hale et al., 2006). The latter activation was abolished when an IAV lacking NS1 (delNS1) was used (Ehrhardt et al., 2006). In addition, stable expression of NS1 alone was able to induce the phosphorylation of Akt at S473, which was sensitive to general PI3K inhibitor (Hale et al., 2006). Thus, the second activation of PI3K in IAV infected cells is mediated by NS1.

The structural and mechanistic basis for PI3K activation by NS1 has been well studied. It is mediated by the interaction of NS1 with the p85 regulatory subunit of PI3K. The interaction is isoform specific, since NS1 interacts only with the β isoform of p85 (Hale et al., 2006; Shin et al., 2007a). The interaction preface between NS1 and p85β has been established by mutational analyses as well as by resolving the crystal structure of the interaction (Hale et al., 2008b; Hale et al., 2006; Hale et al., 2010a; Li et al., 2008).

p85β contains five domains: an SH3-domain, a GTPase activating protein domain, two SH2-domains, and an inter SH2 (iSH2) domain which separates the two SH2 domains (Okkenhaug and Vanhaesebroeck, 2001). The p85 subunit binds to p110 through the iSH2 domain (Dhand et al., 1994; Klippel et al., 1993) where NS1 also binds (Hale et al., 2008b). Tyrosine residue at the position of 89 (Y89) has been reported to be crucial for the interaction and substitution of tyrosine at this position to phenylalanine (Y89F) abrogates the NS1-p85β binding and PI3K/Akt activation (Hale et al., 2006). The crystal structure of the interaction confirmed that the residue Y89 and a proline at 164 (P164) are located at the binding interface with p85β (Hale et al., 2010b). Glutamine residue 142 in NS1 seems to also contribute for the interaction (Hale et al., 2010b; Li et al., 2008). In p85β the crucial amino acid for the interaction is valine at position 573 (Li et al., 2008). Mutation of the corresponding residue on p85α (methionine 582) to valine enables NS1 to interact also with p85α (Li et al., 2008). Crystal structure of the interaction reveals that methionine instead of valine at this position in p85β would be sterically precluded, explaining the strict isoform specificity of NS1-p85 binding (Hale et al., 2010a). The association of NS1 with p85β removes the inhibitory contacts between the p85β and p110 leading to the activation of PI3K (Hale et al., 2008b; Hale et al., 2010a). In addition, NS1 possibly directly stimulates the activity of p110 (Hale et al., 2010b). Mutations in a small, acidic α-helix in NS1 (residues 95-100) that lies adjacent to the activation loop of p110, generated a virus that was unable to produce PIP_3 during the infection, and consequently failed to induce S473 phosphorylation of Akt.

The biological relevance of NS1-mediated PI3K activation for IAV infection is still not clear. As the PI3K signaling is known to mediate cell survival via activation of Akt (Cooray, 2004), the NS1-mediated PI3K activation has been proposed to stimulate cell survival. By using chemical PI3K-inhibitors during virus infection, NS1-mediated PI3K activation was reported to be involved in inhibition of apoptosis in a human A/PR8 and an avian A/FPV/Bratislava/79 IAV strains (Ehrhardt et al., 2007; Zhirnov and Klenk, 2007). Likewise, Shin et al. reported a more proapoptotic phenotype of A/PR8 IAV with an NS1 mutated at residue P164, which is important for p85-binding (Shin et al., 2007a). Several studies have also reported the importance of NS1-mediated PI3K activation for viral replication and virulence. Ehrhardt and colleagues used a chemical PI3K-inhibitor during the infection of cultured cells with A/PR8 and A/FPV IAV strains (Ehrhardt et al., 2006). The inhibition of PI3K resulted in impaired viral propagation. Likewise, by mutating the NS1 residue Y89 in human A/PR8 virus, it was shown that activation of PI3K signaling enhances viral replication in tissue culture and the virulence in a mouse model (Ayllon et al., 2012b; Hrincius et al., 2012). However, the requirement for PI3K activation seems to be viral strain specific, since the Y89F mutation in another human IAV strain, A/WSN, did not have any effect on viral replication or virulence (Ayllon et al., 2012b). Furthermore, mutation in A/Udorn NS1 to block the interaction with p85β and subsequent loss of PI3K activation had no effect on IAV infected cell viability (Jackson et al., 2010).

1.5 SH3 domains

The SH3 domains are short, approximately 50-70 residues long, and they are characterized by their ability to bind to proline-rich peptide modules. They were discovered in 1988 when two groups identified independent regions of sequence similarity between divergent signaling proteins of the Src family of non-receptor tyrosine kinases, the Crk oncogene, and phospholipase C-γ (PLCγ) (Mayer et al., 1988; Stahl et al., 1988). The fact that the domain was found in many different proteins and did not appear to have any enzymatic activity, implied that the domain was modular and possessed independent functions in protein complexes.

SH3 domains are one of the most common modular protein domains found in eukaryotic genomes (Mayer and Saksela, 2004). The human genome has been estimated to contain 296 different SH3 domains (Kärkkäinen et al., 2006). Since one protein may contain up to six SH3 domains, the number for SH3 domain containing proteins is somewhat smaller. SH3 domains are present in many different cellular proteins, including scaffold proteins, adapter proteins and enzymes. They mediate specific protein-protein interactions and they are involved in many important cellular functions, such as regulation of signal transduction, cytoskeletal organization and membrane trafficking (Mayer and Saksela, 2004). Binding of an SH3 domain to its specific ligand recruits proteins to various subcellular locations, assembles multi-protein signaling complexes, and regulates enzyme activities. Many SH3 domain containing proteins also possess other protein-protein interaction domains, such as SH2 (Src homology 2) domains, emphasizing the importance of these proteins in multi-protein complex formation and signal transduction in cells. SH2 domains bind to short phosphotyrosine containing motifs within their target proteins to mediate similar functions as SH3 domains.

1.5.1 SH3 domain structure

Crystallography and NMR studies have revealed the highly conserved three dimensional structure of SH3 domains (Musacchio et al., 1992; Yu et al., 1992). They consist of five β-strands (β_1- β_5) that are arranged to two tightly packed anti-parallel β-sheets. The first β-sheet is composed of β_1, half of the β_2 and the β_5, while the second is formed of the second half of the β_2, β_3 and β_4 -strands. The β-strands are connected by three variable loops, denoted as RT, n-Src, and distal loops as well as by a short 3_{10}–helix. The first two strands (β_1- β_2) are separated by the RT, the β_2- β_3 by the n-Src, the β_3- β_4 by the distal loops, and the last two (β_4- β_5) by the 3_{10}–helix.

The ligand binding site of SH3 domain has a relatively shallow binding surface. It is formed by the conserved hydrophobic residues in β_3- and β_4-strands, N-Src loop and the tip of the RT loop (Musacchio et al., 1992; Yu et al., 1992). The binding surface can be divided into three binding pockets: two hydrophobic pockets, lined mainly by aromatic residues and a specificity pocket, formed by residues from RT and n-Src loops.

1.5.2 SH3 domain ligand binding motifs

1.5.2.1 Typical ligand binding motifs

Early studies identified that the SH3 domains bind to proline-rich peptides (Ren et al., 1993). It was further noticed that SH3 domains bind specifically to two consensus peptides containing ϕP moieties, where ϕ is usually a hydrophobic residue (Kay et al., 2000). SH3 ligand peptide adopts a polyproline-2 (PPII) structure, a left handed helix, which has three residues per turn and is roughly triangular in cross-section (Musacchio, 2002). The base of this triangle sits on the surface of the SH3 domain and the two hydrophobic pockets of the SH3 binding site recognize ϕP dipeptides of the PPII helix, whereas the third, “specificity” pocket binds to a positively charged residue, which flanks the ϕPxϕP core binding motif (see Figure 5). The positively charged residue is typically either lysine (K) or more commonly arginine (R).

The structural studies on SH3-peptide complexes have identified that PPII helix can bind in two orientations with respect to the SH3 domain (Feng et al., 1994; Lim et al., 1994). The peptides that can bind in N to C orientation relative to SH3 domain have been classified as class I ligands, whereas the C to N binding peptides are called class II ligands. The orientation of the peptide depends on the location of a positively charged residue. The consensus sequence for class I ligands is +xϕPxϕP, and for class II ligands ϕPxϕPx+ (where P is proline, + is R or K, ϕ is a hydrophobic residue, and x is any amino acid). Some SH3 domains bind to the ligand peptide only in one orientation, whereas some can bind ligands in both orientations.

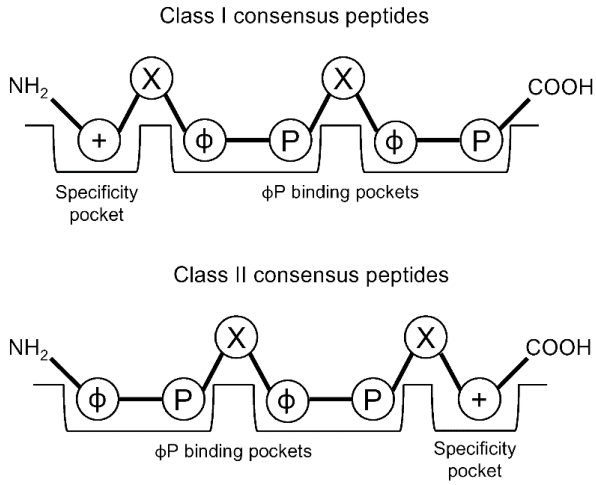

Figure 5. Binding of class I and class II consensus ligands to SH3 domains. After Zarrinpar et al., 2003.

1.5.2.2 Atypical ligand binding motifs

In addition to class I and class II SH3 binding sites, a number of alternative motifs have been identified that lack the core PxxP element (Li, 2005; Mayer and Saksela, 2004). For example, the SH3 domains of Eps8 family members and the N-terminal SH3 domain of Nck1 bind to ligands that do not contain canonical PxxP-motifs. Instead, they have a unique binding preference for PxxDY motif (Kesti et al., 2007; Mongiovi et al., 1999). The first hydrophobic pocket of Eps8L1 SH3 domain is not optimal for binding the conventional PxxP peptides since it is smaller than usually (Aitio et al., 2008). Some SH3 binding motifs even totally lack the proline residues, like the SH3 domains of Fyn and Fyb, which bind selectively to RKxxYxxY consensus in SKAP55 (Src kinase-associated protein of 55 kDa) adaptor protein (Kang et al., 2000). Also, SH3 domains of Fyn and Lck that bind to class I binding motifs, could interact with this motif. Hence, the recognition of RKxxYxxY seems to be similar to the class I consensus recognition sequence. Moreover, several SH3 ligand proteins have been found to contain an R/KxxK/R consensus binding site. For example, the C-terminal SH3 domains of Grb2 and Gads adaptor proteins interact with proteins that has the R/KxxK/R consensus (Berry et al., 2002; Lewitzky et al., 2001; Liu et al., 2003). The R/KxxK/R consensus is sometimes referred as the class III binding motif, since it is found in numerous SH3 ligand proteins (Li, 2005). Many more unconventional binding site sequences have been identified, including the RxxPxxxxP consensus found in the calcium activated potassium channel which mediates binding to the SH3 domain of cortactin (Tian et al., 2006).

1.5.3 Affinity and specificity of SH3 domains

The affinity and specificity of SH3 domains to their ligands is relatively low and the dissociation constant (K_d) normally varies in the 1-200 µM range (Mayer and Saksela, 2004). Since only five residues of the core binding motif have direct contact with the SH3 domain, additional contacts between the variable loops of the SH3 domain and residues outside the core PxxP motif in the ligand play a critical role in determining the specificity and affinity of an SH3 domain. For example, the unusually high affinity (K_d 24 nM) of the

SH3 domain of $p67^{phox}$ phox (phagocyte oxidase) protein with $p47^{phox}$ requires 20 additional residues immediately C-terminal to the PxxP motif in $p47^{phox}$ (Kami et al., 2002). When the additional residues were removed, the affinity of the interaction was turned into a very modest one (K_d 20 μM). Also the avid and highly selective binding of PEP (proline enriched phosphatase) to the SH3 domain of Csk requires additional residues outside the binding motif. Two hydrophobic amino acids, isoleucine and valine, located six residues after the consensus class II PxxP motif in PEP are placed into the specificity pocket of Csk SH3 domain (Ghose et al., 2001). In addition, 3_{10} helix structure positioned after the PxxP motif in PEP helps to orientate the residues into the specificity pocket.

The binding of HIV-1 Nef protein with SH3 domain of Src family kinase Hck provides an example of an SH3 domain interaction where the affinity and specificity both require additional residues outside the PxxP motif in Nef in the ligand and sequence variation in the RT-loop of an SH3 domain. The affinity of Hck SH3 domain to Nef is very high (K_d 0.25 μM), whereas another Src family member, Fyn, has a very modest binding affinity (K_d < 20 μM) for Nef. It was found that a single amino acid at the tip of the RT loop of the Hck SH3 domain compared to Fyn was important for this high affinity binding (Lee et al., 1995). When the corresponding amino acid in the RT-loop of Fyn (arginine) was substituted for isoleucine as in Hck, the affinity of Fyn for Nef was almost as good as that of Hck. In addition, the core PxxP site in Nef was not sufficient for the tight binding as a 12-residue long peptide overlapping the PxxP-site of Nef had only modest binding affinity and specificity towards Hck SH3, suggesting that additional regions in Nef are important for the interaction. The crystal structure of Nef-SH3 interaction revealed that regions distal to the PxxP motif in Nef are important for the binding (Lee et al., 1996). The PxxP motif of Nef is followed by two α-helices forming a hydrophobic pocket that engages the specificity-determining isoleucine residue of the SH3 domain.

1.5.4 Viral proteins as SH3 domain ligands

As SH3 domains are involved in regulation of numerous cellular events, they serve as a potential target for virus to regulate host cell signaling to optimize their replication during the infection. Indeed, several viral proteins have been reported to interact with host cell proteins through their SH3 domains. The first characterized viral SH3 domain ligand protein was the Nef protein of HIV-1 (Saksela et al., 1995). Nef is essential for HIV-1 replication and plays an important role as a pathogenesis factor of HIV-1 infection. The best known cellular activities of Nef in host cell are dependent on its highly conserved SH3 binding motif, which has a consensus PxxPxR (Saksela, 2011). Disruption of the polyproline motif in Nef results in impaired replication. In addition to previously mentioned Nef association with SH3 domains of Src-family kinases Hck and Fyn (Saksela et al., 1995), Nef has also been reported to interact with other family members, namely c-Src, Lck and Lyn. The binding of Nef to Hck is one of the strongest reported interactions between an SH3 domain and its ligand (Lee et al., 1995), whereas the binding of Nef to other SFKs has a modest affinity. The interaction of Nef with Hck results in the activation of the kinase by disrupting the intramolecular interaction between the SH2-kinase linker and the SH3 domain (Moarefi et al., 1997). The intramolecular SH3-ligand interaction is a key mechanism in Src-family kinases. Trible et al. showed that among the Src-family kinases, Lyn and c-Src were also activated by the association of Nef with their SH3 domains (Trible et al., 2006). In contrast, they noticed that Fyn, Lck, Fgr and Yes were

not activated by the interaction. As a matter of fact, the association of Nef with Lck and Fyn has been reported to lead to suppression of the kinase activity (Briggs et al., 2000; Greenway et al., 1996).

The NS5A protein of hepatitis C virus (HCV) is essential for both viral replication and virion assembly (Ross-Thriepland and Harris, 2015). It contains one N-terminal and two C-terminal polyproline motifs and is known to have several host cell interaction partners (Macdonald et al., 2004; Macdonald et al., 2005; Tan et al., 1999). No interaction partners for the N-terminal class I PxxP motif has been found so far. Instead, the two closely spaced class II polyproline motifs near the C-terminus of NS5A have been identified as a binding site for SH3 domains of numerous host cell proteins. The first C-terminal PxxP binds to the SH3 domain of Src-family kinase Lyn, whereas the second C-terminal PxxP has been reported to associate with the SH3 domains of other Src kinases, namely Hck, Lck and Fyn (Macdonald et al., 2004) as well as with the adaptor protein Grb2 (growth factor receptor-bouns protein 2) (Tan et al., 1999). The interaction of NS5A with the SH3 domains of the Src-family kinases results in differential regulation of the kinase activity. While the activity of Hck, Lck and Lyn is suppressed by the interaction, the activity of Fyn is stimulated (Macdonald et al., 2004). The stimulation of Fyn by NS5A binding to its SH3 domain was linked to tyrosine phosphorylation of transcription factor Stat3. Since the constitutive phosphorylation of Stat3 is associated with many tumours, the Fyn mediated phosphorylation of Stat3 was hypothesized to be involved in the development of hepatocellular carcinoma in HCV-infection. The interaction of NS5A with the SH3 domain of Grb2 was reported to disturb vaccinia virus induced phosphorylation of ERK1/2 (extracellular signal-regulated kinases 1 and 2) (Tan et al., 1999). The second C-terminal SH3 binding motif also mediates the association of NS5A with the SH3 domains of amphiphysin II (Masumi et al., 2005), MLK3 (Mixed Lineage Kinase 3) (Amako et al., 2013) and an adaptor protein CMS (Cas ligand with multiple SH3 domains) (Igloi et al., 2015; Mankouri et al., 2009). Interaction with amphiphysin was shown to decrease the phosphorylation of NS5A enabling efficient viral replication, since hyperphosphorylation of NS5A inhibits virus replication (Masumi et al., 2005). HCV establishes a persistent infection, thus it is important for the virus to control apoptosis. Indeed, the interaction of NS5A with the SH3 domain of MLK3, a serine threonine protein kinase that is a member of MAP3K (mitogen activated protein kinase kinase kinase) family, has been linked to inhibition of apoptosis. MLK3 activates p38MAPK which leads to up-regulation of K+ channel (Kv2.1) and apoptosis (Amako et al., 2013; Mankouri et al., 2009). The interaction between the SH3 domain of MLK3 and the second C-terminal polyproline motif of NS5A was demonstrated to prevent this activation of Kv2.1 and oxidative-stress induced apoptosis by disturbing MLK3/p38MAPK signaling (Amako et al., 2013). Finally, association of NS5A with the SH3 domain of CMS disrupts the EGFR (epidermal growth factor receptor) trafficking and ubiquitination (Igloi et al., 2015). EGFR is a key factor for HCV entry and replication (Diao et al., 2012).

1.6 Crk adaptor proteins

The history of Crk proteins starts in 1988 when Mayer et al. isolated the *v-crk* (or *gag-crk*) oncogene from chicken tumor virus number 10 (CT10) retrovirus (Mayer et al., 1988). It was named CT10 regulator of kinase (Crk) for its proposed function, since it was able to increase cellular tyrosine phosphorylation and transform primary chicken

embryo fibroblasts but was lacking any catalytic domain. *v-crk* is a fusion gene of the viral *gag* gene and a cellular gene which encodes two separate domains (see figure 6). Couple of years after the finding of v-Crk, Reichman et al. isolated the cellular homolog of chicken Crk which had a similar structural organization to v-Crk but contained a ~50 aa proline rich linker and an additional C-terminal domain (Reichman et al., 1992). Following the sequencing of cellular Crk in chickens, two isoforms of Crk (CrkI and CrkII) and Crk-like (CrkL) were isolated (Matsuda et al., 1992), and the family of Crk adaptor proteins are considered to comprise these three members: CrkI, CrkII and CrkL. CrkI and Crk II are alternatively spliced products from one gene (Matsuda et al., 1992), whereas CrkL is encoded by a distinct gene and shares high sequence homology with CrkII (Galletta et al., 1999; ten Hoeve et al., 1993).

1.6.1 Stucture and binding specificities of Crk proteins

The Crk proteins are relatively small adaptor proteins lacking any enzymatic activity (Feller, 2001). The predicted molecular masses for Crk family proteins are 28 kDa for CrkI, 40 kDa for CrkII, and 36 kDa for CrkL. They possess one N-terminal SH2 domain followed by one (in CrkI) or two (in CrkII and CrkL) SH3 domains (see Figure 6). The SH3 domains are called the N-terminal (nSH3) and C-terminal (cSH3) according whether they are more N- or C-terminally located. The nSH3 and cSH3 domains are separated by an approximately 50 residue long linker region. Compared to CrkL SH2, CrkI and CrkII SH2 domain possess an extra stretch of 17 aas that contain a proline-rich region (Anafi et al., 1996). The overall sequence identity of CrkII and CrkL is 56 %, and in the structural domain regions 72 % (Kobashigawa and Inagaki, 2012).

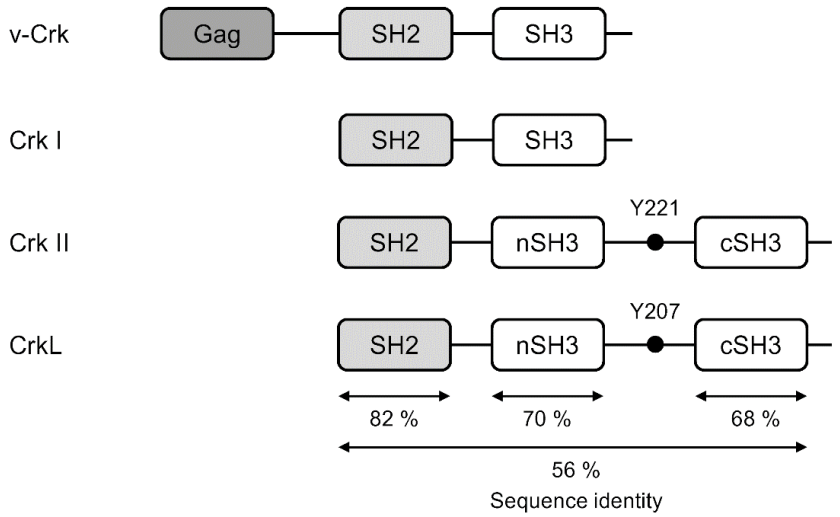

Figure 6. Schematic representation of the domain structure of the Crk adaptor proteins. Sequence identities are shown for CrkII and CrkL. After Birge et al., 2009 and Kobashiwaga and Inagaki, 2012.

The ligand binding specificities of the SH2 and nSH3 domains of CrkII and CrkL are similar. For the SH2, the preferred binding motif is pYxxP (where pY means phosphorylated tyrosine residue) (Songyang et al., 1993). Multiple pYxxP motifs are found in Crk SH2

binding proteins like paxillin (Birge et al., 1993), p130Cas (Sakai et al., 1994), and c-Cbl (Ribon et al., 1996). CrkII SH2 domain has also been reported to bind a pYxxL motif in the cell cycle regulator Wee1 (Smith et al., 2000). In general, the nSH3 of Crk proteins bind to class II polyproline motifs, and the consensus binding motif is PxxPxK (Knudsen et al., 1994; Tanaka et al., 1994). The lysine (K) residue as a positive residue in the consensus was found to be critical for high affinity binding of C3G to the nSH3 domain of Crk proteins (Knudsen et al., 1995). Changing the lysine to arginine, which normally is the preferred positive residue in SH3 binding motifs, strongly reduced the affinity of C3G-derived peptide for the nSH3 domain. The remarkable affinity and selectivity created by the lysine residue over arginine was explained by the unique structure of the nSH3 domain specificity-pocket, where three acidic residues is involved in forming hydrogen bonds with lysine (Wu et al., 1995). In contrast, the arginine side chain is not well ordered and does not adopt an optimal conformation for hydrogen bonds. The cSH3 domain is an atypical SH3 domain, since the conserved residues in the hydrophobic polyproline binding pockets are replaced by polar residues, and so far no polyproline containing binding partners have been identified for the cSH3 (Muralidharan et al., 2006). Instead, cSH3 is involved in regulation and nuclear export of the Crk proteins (see below).

1.6.2 Regulation of Crk proteins

The linker region between nSH3 and cSH3 domains of CrkII and CrkL contains a tyrosine residue (Y221 in CrkII and Y207 in CrkL; Figure 6) that is important for the regulation of these proteins. When phosphorylated by tyrosine kinase c-Abl, the tyrosine in the linker region of CrkII and CrkL binds to its own SH2 domain to form a closed structure that prevents the SH2 domain from binding to its tyrosine phosphorylated ligand proteins (de Jong et al., 1997; Feller et al., 1994; Kobashigawa et al., 2007; Rosen et al., 1995). In addition, the closed structure prevents the binding of nSH3 domain of CrkII ligands, but in CrkL the nSH3 domain is fully accessible (Jankowski et al., 2012).

NMR studies with chicken CrkII revealed another auto-inhibition regulation method by a *cis-trans* isomerization at a glycine-proline (G237-P238) peptide bond located at the cSH3 boundary (see figure 7) (Sarkar et al., 2007; Sarkar et al., 2011). In the *cis* conformer, nSH3 and cSH3 are able to interact with each other when three residues of cSH3 (P238, F239 and I270) occupy the PPII binding site on nSH3 in a manner similar to a PPII peptide. This prevents the binding of nSH3 domain to its natural ligands. In contrast, the *trans* conformer adopts an open state where the PPII binding site on nSH3 is able to bind PPII ligands. In human CrkII, residues 224-237 (called the inter SH3 core; ISC) within the cSH3 form contact the SH2 and both SH3 domains (Kobashigawa et al., 2007). These contacts arrange the three domains into a compact structure and represses the interaction of nSH3 domain with its ligands.

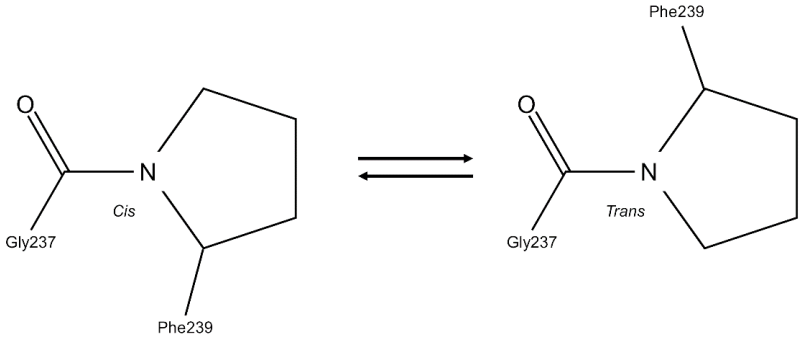

Figure 7. Schematic representation of *cis-trans* isomerization in Crk II. After Sarkar et al., 2007.

The solution NMR structure of CrkL revealed that the overall domain organization in CrkL is very different from CrkII (Jankowski et al., 2012). The binding site of SH2 domain is partially masked in CrkL, while in CrkII it is fully accessible. In addition, the cSH3 of CrkL does not seem to have similar auto-inhibition activity as in CrkII. In CrkL the cSH3 domain is mobile and does not interact with any of the other domains. Thus, the binding site of nSH3 domain is fully accessible in CrkL, while in CrkII it is masked by the ISC.

Finally, the cSH3 domain has been shown to possess a positive regulatory role as well. In contrast to conventional SH3 domains, cSH3 has a conserved PNAY-motif in the RT-loop (Reichman et al., 2005). The tyrosine residue, Y251, within the PNAY-motif was shown to be phosphorylated by c-Abl (Reichman et al., 2005; Sriram et al., 2011). Phosphorylation at Y251 enables the association of the c-Abl SH2 domain with this site, which enhances the activation of Abl. In contrast, Jankowski et al. reported that in CrkL only one tyrosine residue is phosphorylated by c-Abl *in vitro* (Jankowski et al., 2012).

1.6.3 Nuclear import and export of Crk proteins

Crk proteins are predominantly found in the cytoplasm, but they also are able to enter the nucleus, although they do not seem to have an NLS. Therefore, they are thought to be imported to the nucleus through their interaction with other proteins that contain an NLS (Kar et al., 2007). The interaction of nSH3 with Wee1, DOCK180 and c-Abl has been reported to mediate the nuclear translocation of CrkII (Kar et al., 2007). For example, in CrkII-Wee1 complex, the NES of CrkII is masked, retaining CrkII in the nucleus. On the contrary, the nuclear export of Crk proteins is an active process, which is mediated by the interaction of NES, located in cSH3 domain, with a nuclear export receptor Crm1/exportin (chromosome maintenance region-1) (Smith et al., 2002). Harkiolaki et al. reported that CrkL cSH3 can exist in monomeric or dimeric conformation (Harkiolaki et al., 2006). The crystal structure of the cSH3 revealed that the NES is mostly buried under the domain surface in both conformations. However, upon dimer/monomer transition, partial unfolding of the cSH3 exposes the NES and the complex with Crm1/exportin can form.

1.6.4 Biological function of Crk proteins

The members of the Crk family are involved in several signal transduction pathways downstream of a wide range of receptors (Birge et al., 2009; Feller, 2001). Like other

adaptor proteins, they physically bridge tyrosine phosphorylated proteins to various intracellular signaling pathways. The SH2 domain serves as an input pathway by binding to activated receptors and the signal is then transmitted through the interaction partners of nSH3 domain. Crk proteins have been reported to associate with numerous cellular proteins through their SH2 and SH3 domains and they have important regulatory roles in numerous cellular signaling processes, including cell adhesion, growth, differentiation, proliferation, transformation, and apoptosis.

Although Crk proteins are ubiquitously expressed, and their SH2 and SH3 domains are highly homologous with similar binding preferences, they seem to have distinct, non-overlapping roles during embryonic development. Knockout of CrkI/II or CrkL leads to different developmental defects and the mice die perinatally (Guris et al., 2001; Park et al., 2006). Mice lacking the CrkI and CrkII die perinatally due to defects in cardiovascular and craniofacial development (Park et al., 2006), whereas the CrkL null mice have defects in neural crest and cardiac development (Guris et al., 2001). Furthermore, in mouse embryonic fibroblasts (MEFs) where CrkII or CrkL has been depleted the other protein does not compensate the loss of the other by overexpression, again suggesting that each of the two genes has essential biological functions that cannot be replaced by the other gene. Mice that lack CrkII but still expresses CrkI develop normally, suggesting that CrkI can compensate the loss of CrkII in development, but they die within a few days after birth by unknown mechanisms (Imaizumi et al., 1999).

However, Crk proteins have been shown to have essential overlapping roles as well. Tissue-specific contribution of Crk proteins has been studied by generating floxed alleles of *Crk* and *CrkL*. The lack of both CrkI/II and CrkL in skeletal muscle led to severe defects in the neuromuscular synapse, whereas mice lacking only one of the proteins did not show any defects (Hallock et al., 2010). A recent study demonstrated that Crk proteins play overlapping roles in maintaining cell structure and motility in MEF cells (Park and Curran, 2014). The lack of both genes resulted in smaller cell size, while the lack of only *Crk* or *CrkL* did not have any effect on cell morphology.

1.6.4.1 Crk in PI3K signaling

Crk proteins have been reported to participate in the regulation of PI3K signaling. The viral oncoprotein v-Crk activate PI3K/Akt pathway in CEF (chicken embryonic fibroblast) cells (Akagi et al., 2000). Both SH2 and SH3 domains are important for the activation, since SH2- or SH3-mutant forms of v-Crk were not able to activate the pathway. The v-Crk SH2 domain was demonstrated to be involved in PI3K activation by mediating the activation of Src family kinases which in turn phosphorylate focal adhesion kinase (FAK) (Akagi et al., 2002). Tyrosine phosphorylated FAK then associates with the SH2 domain of p85 regulatory subunit of PI3K, which was shown to be essential for the pathway activation. The v-Crk SH3 domain was demonstrated to participate in the induction of the pathway by activating H-Ras, a member of Ras family of small G proteins, which in turn binds to the p110 catalytic subunit of PI3K and may activate it. The activation of H-Ras was shown to be mediated by binding of SOS (son of sevenless), a guanine nucleotide exchange factor, to the SH3 domain of v-Crk.

In addition, Crk proteins have been reported to directly interact with the p85 regulatory subunit of PI3K through the Crk nSH3 domain and a proline-rich motif in p85 (Gelkop et al., 2001; Sattler et al., 1997). The interaction was shown to be involved in PI3K

regulation upon immune cell activation, where a trimeric complex of Cbl, a ubiquitin ligase, CrkII and p85 is formed (Gelkop et al., 2001). The Crk-p85-Cbl complex has also been shown to be involved in oncogenic signaling in chronic myeloid leukemia (Sattler et al., 1996). Binding of CrkL SH3 domain to Bcr-Abl fusion protein resulted in phosphorylation of CrkL and subsequent binding to the SH2 domain of Cbl. Cbl is then phosphorylated which leads to its association with p85.

1.6.4.2 Crk in apoptotic pathways

CrkII was shown to be required for apoptosis in *Xenopus* egg extract by activating caspases (Evans et al., 1997). Depletion of CrkII from egg extracts prevented apoptosis, and further studies indicated that the cell cycle regulatory protein Wee1 interacts with CrkII SH2 domain (Smith et al., 2000). The involvement of CrkII-Wee1 interaction in apoptosis was further studied in mammalian cells (Smith et al., 2002). This study demonstrated an NES sequence found in the cSH3 domain of CrkII. It was found to mediate the binding of CrkII to Crm1 and subsequent nuclear export of CrkII. Mutation of this sequence enhanced the association of CrkII with Wee1. Furthermore, the proapoptotic activity of CrkII was increased when the NES was impaired, further implying that the proapoptotic function of CrkII depends on nuclear localization. Indeed, specific targeting of CrkII by adding an NLS was shown to spontaneously activate apoptosis (Kar et al., 2007).

CrkI and CrkII have also been shown to be required for ER (endoplasmic reticulum) stress-induced apoptosis, which is mediated by the mitochondrial apoptotic pathway (Austgen et al., 2012). CrkI/CrkII KO MEFs were resistant to ER stress-induced apoptosis. In addition, ER stress was found to induce proteolytic cleavage of CrkI/II by a cysteine protease. This about 14 kDa N-terminal fragment was further shown to have enhanced apoptotic activity when transiently expressed. The proapoptotic function of the fragment was found to be due to CrkI/II binding to an antiapoptotic Bcl-2 family protein, Bcl-XL, upon ER stress induction. The interaction of CrkI/II with Bcl-XL is mediated by a BH3 (Bcl2 homology 3) death domain found in the 14 kDa N-terminal fragment. Approximately 9-12 amino acid long BH3 domain is involved in activation or inhibition of proapoptotic or antiapoptotic proteins, respectively (Lomonosova and Chinnadurai, 2008).

2 AIMS OF THE STUDY

The aim of this doctoral thesis was to study influenza A virus NS1 protein and its host cell SH3 domain interactions. More specifically the aims were:

1. to characterize a putative SH3 binding motif in IAV NS1 protein, its host cell SH3-domain interaction partners, and the functional role of these interactions.
2. to study the role and molecular mechanism of Crk-NS1 interaction for the enhanced PI3K activation by NS1 protein.
3. to study the subcellular localization of the Crk-NS1 complex and its role in the host cell.

3 MATERIALS AND METHODS

3.1 Cell culture and transfections (I-III)

Human embryonic kidney fibroblast (293T) cell line (**I,II**) was purchased from ATCC, human hepatocellular carcinoma (Huh7) cell line (**I-III**) was obtained from Mark Harris (University of Leeds, England) , and human lung adenocarcinoma epithelial (A549) cell line (**I,III**) was received from Ilkka Julkunen (THL, Helsinki, Finland). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Aldrich) supplemented with 4500 mg/L of glucose, 10% fetal bovine serum (FBS) (Gibco), 0.05 mg/ml penicillin, 0.05 mg/ml streptomycin (Sigma Aldrich), and 1 mM L-glutamine (Sigma Aldrich) and were maintained in a humidified atmosphere in the presence of 5% CO_2 at 37 °C.

Transient transfection of 293T cells was performed by the calcium phosphate precipitation method. Briefly: 10-20 µg of plasmid DNA was mixed with 250 µl of water, and 250 µl of 2.5 M $CaCl_2$ was added and mixed. The plasmid mix was added dropwise in 500 µl of 2xHeBS (274 mM NaCl, 10 mM KCl, 1,4 mM Na_2HPO_4, 15 mM glucose, 42 mM Hepes, pH 7.05) while vortexing. The transfection mix was kept in RT for 20-30 minutes and added dropwise on top of the cells in 10 cm culture plate and the medium was changed after 12 hours. Huh7 and A549 cells were transfected with Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions.

3.2 Plasmid constructs

3.2.1 Eukaryotic expression vectors (I-III)

The cDNA fragment for A/Mallard NS1 protein was cloned from the total cellular RNA of cells infected with A/mallard/Netherlands/12/2000 (H7N3) IAV strain by standard methods. Synthetic cDNA encoding A/Brevig Mission/1/18 (H1N1) IAV strain, so called the Spanish Flu, NS1 protein (A/Brevig) was purchased from GENEART. The cDNAs for NS1 proteins of A/WSN/33 (H1N1) (A/WSN) and A/Udorn/72 (H3N2) (A/Udorn) IAVs were obtained from Ilkka Julkunen (THL, Helsinki, Finland). The cDNAs for all NS1 proteins were cloned into the pEBB vector (Tanaka et al., 1995) containing an N-terminal myc epitope tag. Codon changes in NS1 gene were generated by standard overlap PCR mutagenesis. In NS1-Cyto, the NLS1 was mutated (R38A, K41A) and a NES from MAPKK1 (LQKKLEELEL) was inserted before the NS1 coding sequence. The cDNA for CrkI, CrkII and CrkL was cloned into the pEBB vector with a C-terminal biotin acceptor domain (PP-domain) from PinPoint-Xa1 T-vector (Promega). The full-length cDNA for p85β was purchased from the Genome Biology Unit (MGC-collection, University of Helsinki) and cloned in to the pEBB vector with an N-terminal HA epitope tag or PP-domain. The codon changes to p85β sequence were done by overlap PCR mutagenesis. The NS1 and p85β mutations used in this study are summarized in Table 2.

Fluorescent fusion proteins were generated by fusing a red fluorescent protein, mCherry, to the N-terminus of A/Mallard NS1 and A/Mallard NS1-Cyto, and a green fluorescent protein, eGFP, to the N-terminus of CrkII and CrkL. The ISRE-Luc reporter plasmid was obtained from James E. Darnell Jr. (Rockefeller University, New York) and contains an interferon-stimulated response element (ISRE) -containing fragment in front of a minimal thymidine kinase promoter driving firefly luciferase expression. *Renilla*

luciferase cDNA from pRL-null vector (Promega) was cloned into pcDNA3.1 vector to create pcDNA-Renilla.

Table 2. NS1 and p85β mutants used in this study.

Mutation	Acronym	Functional consequence	Study
A/Mallard or A/Brevig NS1			
P212A,P215A	AxxA/M1	Disrupts the SH3 binding consensus	I,II
P213A, P216A	M2	No effect	I
K217E	M3	Disrupts the SH3 binding consensus	I
K217R	M4	Alters the specificity of SH3 binding site	I
P215T	M5	Disrupts the SH3 binding consensus,	I
		mimics the human IAV NS1 sequence	I
Y89F		Prevents binding to p85β	II
R38A, K41A + additional NES	NS1-Cyto	Prevents nuclear localization	III
p85β			
V573M		Prevents binding to NS1	II
P294A,P297A		Prevents binding to Crk nSH3 domain	II

3.2.2 Bacterial expression vectors (I,II)

The GST (Gluthathione S-transferase) and MBP (maltose binding protein) –NS1 constructs were generated by cloning the cDNAs of A/Mallard and A/Brevig into pGEX-4T-1 vector (GE Healthcare) and pMAL-c2x (New England Biolabs), respectively. GST-PP-SH3 constructs for CrkII, CrkL, p85α, p85β, and Eps8L1 SH3 domains were generated by transferring the codon optimized cDNAs from the human SH3 domain library (Kärkkäinen et al., 2006) into pGEX-4T-1 which had a PP-domain inserted between the GST and multiple cloning site. Coding sequence for the peptide CLNCFRPLPPLPPPPR was cloned into the pMAL-c2x vector to generate the construct for p85α-BP (p85α binding peptide).

3.3 Viruses and virus infections (I,III)

The wt IAV strains used in this study were: A/mallard/Netherlands/12/2000 (H7N3), A/Udorn/72 (H3N2), and A/WSN/1933 (H1N1). The recombinant IAVs were generated as previously described (Neumann et al., 1999). The background virus for the recombinant IAV viruses used in this study was A/WSN/1933. The NS-segment originated either from A/WSN/1933 or A/mallard/Netherlands/12/2000 virus strains. The codon changes to the NS1 sequence in the NS-segment were done by overlapping

PCR mutagenesis. The recombinant IAVs and their mutations used in study **III** are summarized in Table 3.

IAVs were propagated in 11-day-old embryonated chicken eggs at 34 °C for 3 days. Virus infections of A549 cells were carried out in normal medium supplemented with 2 % FBS and in the presence of 5 µg/ml of TPCK-treated trypsin (Sigma Aldrich) for 20-24 hours at a multiplicity of infection (MOI) of 0.5 to 5 depending on the assay. To metabolically label virus-infected cells, the medium was changed 4 hours post-infection to a medium that was supplemented with [^{35}S]Met (GE Healthcare).

Table 3. Recombinant IAVs used in study III.

Recombinant virus name	Backbone virus	Description
A/WSN-NS1$^{Mallard(wt)}$	A/WSN/33	A/Mallard NS1 wt
A/WSN-NS1$^{Mallard(K217E)}$	A/WSN/33	A/Mallard NS1 K217E (SH3-binding disrupting mutation)
A/WSN-NS1$^{WSN(wt)}$	A/WSN/33	A/WSN NS1 wt
A/WSN-NS1$^{WSN(T215P)}$	A/WSN/33	A/WSN NS1 T215P (SH3-binding competent mutation)

3.4 Antibodies (I-III)

The following primary antibodies were used in this study: mouse anti-Myc (Sigma-Aldrich), mouse anti-HA (Santa Cruz Biotechnology), mouse anti-α-tubulin (Sigma-Aldrich), rabbit anti-Histone H3 (Cell Signaling Technologies), mouse anti-CrkL (Millipore), mouse anti-Crk (BD Transduction Laboratories), mouse anti-Akt (Cell Signaling Technologies), rabbit anti-phospho Akt (Ser473) (Cell Signaling Technologies), mouse anti-p85β (AbD Serotec) mouse anti-phosphotyrosine (PY20, Santa Cruz Biotechnologies), rabbit anti-NP (Ronni et al., 1995), and guinea-pig anti-NS1 (Melen et al., 2007). The secondary antibodies for Western blotting were purchased from LI-COR Biotechnology: IRDye680CW goat anti-mouse, IRDye800CW goat anti-mouse, IRDye680 goat anti-rabbit, IRDye800CW goat anti-rabbit, IRDye800CW rabbit anti-guinea-pig, Streptavidin IRDye680CW, and Streptavidin IRDye800CW. AlexaFluor 488 goat anti-guinea-pig (Abcam) and AlexaFluor 546 anti-mouse (Molecular Probes) were used as secondary antibodies for immuno fluorescence staining and nuclei were stained with Hoechst (Sigma Aldrich).

3.5 Protein sequence alignment (I)

The amino acid sequences for different NS1 proteins were obtained from the NCBI Influenza Virus Resource data base. The sequence alignment of multiple NS1 proteins was done with ClustalW software.

3.6 Recombinant protein production, purification and phage screening (I,II)

The fusion proteins with GST and MBP were expressed in *E. coli* strain BL21 and purified by using glutathione-Sepharose 4B agarose (GE Healthcare) or amylose resin (New England Biolabs), respectively. The human proteome SH3 domain library used in this study is characterized and the phage screening was done as described (Kärkkäinen et al., 2006).

3.7 Protein precipitation and Western blot (I-III)

For immunoprecipitation and protein pulldown experiments, transfected or infected cells were collected and lysed in 1 % NP40 lysis buffer (150 mM NaCl; 50 mM Tris-HCl, pH 7.9; 1 % NP40) in the presence of protease and phosphatase inhibitors. Cell lysates were used for immunoprecipitation with anti-CrkL (**I-III**) or anti-HA (**II**) antibodies coupled to Dynabeads protein G magnetic beads (Invitrogen) or for streptavidin pulldown (**I,II**) with Tetralink tetrameric avidin resin (Promega) (**I**) or Dynabeads streptavidin coated magnetic beads (Invitrogen) (**II**).

For total protein samples, the protein concentrations were determined by Bio-Rad Protein Assay (Bio-Rad). Immunocomplexes and total protein samples were separated by SDS-PAGE and transferred onto nitrocellulose membrane (Bio-Rad) for subsequent immunoblotting with specific antibodies. Western blots were visualized with the Odyssey infrared imaging system (LI-COR Biosciences).

3.8 Detection of phosphorylated proteins (I-III)

To determine the phosphorylation status of Akt, Huh7 cells were transfected with different NS1 expression constructs (I-III) alone or together with Crk or CrkL expression constructs (**II**). Transfected cells were serum starved with a medium containing 2 % FBS for 12 hours prior the collection 48 hours post-transfection. Collected cells were lysed with NP40 lysis buffer supplied with protease and phosphatase inhibitors and subjected to Western blotting with anti-pAkt (S473) antibody. To study the tyrosine phosphorylation of nuclear proteins (**III**), A549 cells were infected with recombinant IAVs for 24 hours. To enhance the accumulation of tyrosine phosphorylated proteins, cells were treated with phosphotyrosine phosphatase inhibitor pervanadate for 10 minutes. The fractions were then prepared as described in section 3.10 and the lysates were subjected to Western blot analysis with anti-pTyr antibody.

3.9 Dual-luciferase assays (I)

ISRE-Luc reporter plasmid was transfected together with pcDNA-Renilla and NS1 expression constructs or empty vector into Huh7 cells. 22 hours after transfection, the cells were treated with 100 IU/ml of IFNβ (Betaferon, Schering) for 7 hours to induce the reporter gene. The luciferase activities were measured using a dual-luciferase assay (Promega). Renilla luciferase construct was used as an internal control for transfection.

3.10 *In vitro* protein binding assays (I,II)

Purified recombinant MBP-fusion proteins (**I**) were coated on 96-well plates (200 ng/well) and blocked with 1.5 % BSA in TBS (Tris-buffered saline) for 1 hour. The wells were washed twice with TBST (TBS + 0.05 % Tween 20). Bacterial purified GST-PP-fused

SH3 domains were added in 2-fold dilutions and incubated for 1.5 hours. After three washes with TBST, the wells were incubated with streptavidin-biotinylated horseradish peroxidase complex (GE Healthcare) in TBS for 1 hour. The wells were again washed for three times with TBST and 50 µl substrate reagent ABTS Single Solution (Invitrogen) was added. The absorbance was measured 20 minutes later at 405 nm.

To examine the formation of the trimolecular complex of NS1, Crk and p85β *in vitro*, PP-tagged p85β was purified from 293T transfected cells with Dynabeads streptavidin coated magnetic beads (Invitrogen) (**II**). Bacterial purified, GST-fused NS1 and CrkL-SH3 proteins were mixed together with purified p85β and the formed complexes were analyzed with Western blotting.

3.11 Cell fractionation (III)

A549 cells were infected with influenza A viruses at an MOI 2 for 24 hours. The cells were scraped into 500 µl of ice cold Buffer A (20 mM Tris-HCl, pH7.5; 100 mM NaCl; 300 mM sucrose, 3 mM $MgCl_2$) supplemented with 0.5 % Triton X-100 (Sigma Aldrich). After 10 minutes of incubation on ice, the nuclei were pelleted at 800xg for 10 minutes. The cytoplasmic fraction (C) was collected and was centrifuged for an additional 15 minutes at 16100xg. The nuclear pellet was washed once with Buffer A +0.5 % Triton X-100 and twice with Buffer A. The nuclear proteins were collected by dissolving the nuclear pellet into Buffer B (20 mM Tris-Hcl, pH 8.0; 500 mM NaCl, 2 mM EDTA, pH8.0; 0.1 % NP40) and sonicating for 3 seconds. The nuclear extract (N) was cleared by centrifugation at 16100xg for 15 minutes.

3.12 Immunostaining and imaging (III)

A549 cells grown on coverslips were infected with IAVs at an MOI of 0.5 for 20 hours. The cells were then fixed with ice cold methanol for 10 minutes at -20 °C and permeabilized with 0.1 % Triton X-100 for 10 minutes. The cells were blocked with 5 % BSA and 10 % FBS in PBS. NS1 and CrkL were stained with guinea-pig anti-NS1 and mouse anti-CrkL antibodies, respectively, for 40 minutes in the blocking buffer. After three washes, the cells were incubated with secondary antibodies AlexaFluor 488 goat anti-guinea pig or AlexaFluor 546 goat anti-mouse and washed again three times. The nuclei were stained with Hoechst for 5 minutes. The cells were visualized with Leica TCS SP8 confocal microscope scanning the channels sequentially. Open source software Fiji was used to analyze the mean intensities of the CrkL fluorescence signal (Schindelin et al., 2012).

4 RESULTS

4.1 Novel SH3 domain interaction partner for IAV NS1 protein (I-III)

4.1.1 Characterization of a novel interaction partner for NS1 protein (I-III)

Sequence analysis of different viral proteins revealed that the NS1 protein of the Spanish Flu IAV (A/Brevig Mission/1/18; A/Brevig) contains a potential class II SH3 domain binding motif near the C-terminus of the protein sequence (see fig. 1A in **I**). The SH3 binding motif was noted to be very common among avian isolated IAVs but only occasionally found in NS1 proteins of human IAVs (see fig. 1B in **I**). The A/Brevig and many avian isolated NS1 proteins contain a consensus sequence of PxϕPx+, whereas in most human isolated IAV NS1 proteins the second proline has been substituted with threonine (PxϕTPx+). In addition, this SH3 binding sequence motif is not present in the NS1 proteins of IAV strains commonly used in laboratory studies, such as A/WSN/33 (H1N1), A/PR/8/34 (H1N1), and A/Udorn/72 (H3N2) (see fig 1B in **I**).

To identify possible SH3 interaction partners for NS1, we expressed the NS1 proteins of A/Brevig and A/Mallard as GST-fusion proteins and subjected them to affinity screening with human SH3 phage display library. An increased binding of SH3 domain clones to both NS1 proteins was observed as compared with the plain GST protein that was used as a control. The identity of NS1-selected phagemids was determined by sequencing, and more than 90 % of the phages contained the nSH3 domain of the adaptor proteins CrkII or CrkL. To confirm the interaction of NS1 with Crk SH3 domain found with phage screening, we expressed recombinant GST-PP-fusion proteins of the nSH3 domains of CrkII and CrkL. Since NS1 has been reported to interact with the SH3 domain of p85 regulatory subunit of PI3K (Shin et al., 2007a), we also generated GST-PP-fusion proteins of SH3 domains of p85α and p85β. As a negative control, we used the SH3 domain of Eps8L1, which prefers ligands containing PXXDY-motif. The A/Mallard and A/Udorn NS1 proteins were expressed as MBP-fusion proteins. *In vitro* protein binding assay with these proteins revealed the avid binding of the nSH3 domain of CrkII and CrkL to the A/Mallard NS1 (see fig. 2A in **I**), which was in line with our phage screening result. In contrast, the A/Udorn NS1, which lacks the consensus SH3 binding motif, did not show any measurable binding to the CrkII and CrkL SH3 domains (see fig. 2B in **I**). In addition, neither of the NS1 proteins showed binding to the SH3 domains of p85α, p85β, or to the negative control Eps8L1 (see fig. 1A and B in **I**).

To study Crk-NS1 interaction with full-length proteins, we transfected PP-tagged (biotin acceptor domain) expression constructs of CrkII and CrkL together with myc-tagged NS1 proteins of A/Brevig, A/Mallard, or A/Udorn. While A/Brevig and A/Mallard NS1 proteins readily co-precipitated with both CrkII and CrkL, A/Udorn NS1 failed to co-precipitate with either one of them (see fig. 3 in **I**). To further characterize the capacity of all three Crk family members, CrkI, CrkII and CrkL, to interact with NS1 proteins, we co-transfected different Crk-proteins together with A/Mallard NS1-wt or its SH3 binding incompetent mutant (AxxA; P212A, P215A). We could not see any differences of the binding capacity of A/Mallard NS1 wt to CrkI, CrkII or CrkL, while the AxxA-mutation of A/Mallard NS1 totally abrogated the binding (see fig. 1B in **II**).

To extend these finding to IAV infection model, we used an avian IAV strain, A/Mallard and a typical human IAV strain, A/Udorn to infect A549 cells. The NS1 protein from

A/Mallard infected cells readily co-precipitated with endogenous CrkL, whereas the NS1 protein from A/Udorn infected cells did not (see fig. 4 in **I**). In addition, we generated a set of recombinant viruses (see fig. 1A in **III**). The viruses express the NS1 protein from either A/WSN or A/Mallard IAV strains. Mutation to the NS1 gene of A/Mallard (K217E) generated an SH3-binding deficient NS1 protein, whereas the mutation T215P in A/WSN NS1 gene transformed the A/WSN NS1 protein from SH3 binding incompetent to SH3 binding competent. We immunoprecipated endogenous CrkL from cells infected with these recombinant IAVs and examined NS1 co-precipitation by Western blotting. As expected based on our earlier results, A/Mallard NS1-wt was seen to co-precipitate with CrkL, while the mutant, A/Mallard NS1-K217E, did not (see fig. 1B in **III**). Likewise, the mutant A/WSN NS1-T215P was observed to co-precipitate with CrkL, but the A/WSN-wt was not (see fig. 1C in **III**).

To further characterize the critical amino acids involved in association of class II SH3 binding motif containing NS1 with Crk proteins, we generated a series of point mutation variants of the binding site in A/Brevig NS1 (see fig. 5A in **I**). We transfected these mutant variants into cells and performed immunoprecipitation of endogenous CrkL protein. While the wt A/Brevig NS1 co-precipitated with CrkL, the mutant variants with class II binding motif disrupting mutation (M1; AxxA and M3; PxxPx-) could not be co-precipitated with CrkL (see fig. 5B in **I**). Likewise, the mutation T215P (M5), mimicking the sequence of A/Udorn NS1, could not be co-precipitated with CrkL. In addition, swapping the lysine residue to arginine in the consensus sequence in A/Brevig NS1 (M4) diminished the binding to CrkL for ~50 %, which is in agreement with the binding preference of nSH3 domain of Crk proteins to sequences with lysine as a positively charged residue (Knudsen et al., 1995).

4.1.2 Functional role for the Crk-NS1 interaction (I,II)

To study the biological relevance of Crk SH3-NS1 interaction, we tested the activities of A/Brevig and A/Mallard NS1 proteins and their SH3 binding-deficient mutants in two different cellular functions found in the literature for NS1 protein. To test the ability of these NS1 proteins to repress the expression of an interferon-stimulated gene, we co-transfected cells with the ISRE-Luc (interferon-stimulated response element-containing fragment) reporter plasmid together with an empty control expression vector or vector expressing NS1 of A/Brevig, A/Mallard, or A/Udorn, or the SH3-binding mutant versions of A/Brevig (A/Brevig NS1-AxxA) and A/Mallard (A/Mallard NS1-AxxA). After the induction of the reporter vector with IFNβ, the luciferase activity was measured. All NS1 proteins, including the SH3-binding mutants of A/Brevig and A/Mallard were able to inhibit the luciferase expression from ISRE-Luc reporter vector compared to the control where an empty expression vector was transfected (see fig. 6A in **I**). This indicated that the binding of NS1 to Crk and CrkL SH3 domains was not required for the capacity of IAV NS1 proteins to inhibit interferon-stimulated gene expression.

To test the effect of the Crk-NS1 interaction to another known function for NS1, the activation of PI3K pathway, we transfected the same set of NS1 proteins and their mutants and analyzed the ability of these proteins to activate the PI3K signaling by determining the phosphorylation status of Akt (pAkt S473) by Western blotting. The levels of pAkt were significantly higher in cells transfected with A/Brevig or A/Mallard NS1-wt compared with the NS1 constructs lacking the SH3 binding motif either naturally,

as in A/Udorn and A/WSN or by mutation A/Brevig NS1-AxxA and A/Mallard NS1-AxxA (see fig. 6B in **I**). Thus, the SH3 binding motif provides the NS1 with enhanced capacity to induce PI3K activation compared to NS1 proteins lacking the binding site.

Tyrosine residue at position 89 in NS1 has been reported to be critical for NS1- p85β interaction, and NS1-Y89F mutant is not able to interact with p85β or to activate PI3K signaling (Hale et al., 2006). To further analyze the amino acid residues important for the enhanced PI3K activation by SH3 binding competent NS1 proteins, we generated Y89F-mutant construct of A/Mallard NS1. Cells were transfected with A/Mallard NS1-wt, NS1-AxxA (SH3 binding incompetent mutant), or NS1-Y89F (p85β binding mutant), and the phosphorylation status of Akt was examined. While the expression of A/Mallard NS1-AxxA showed reduced PI3K activation compared to the NS1-wt, the Y89F mutation totally abrogated the induction (see fig. 2A in **II**). Thus, while the SH3 binding capacity potentiates the PI3K activation by NS1, the binding of NS1 to p85β through Y89 is indispensable for the activation.

4.1.3 Trimeric complex formation upon Crk-NS1 interaction (II)

In addition to the Crk-NS1 interaction reported here, both NS1 and Crk have been described to interact with p85 (see fig. 3A in **II**). The Crk-p85 interaction has been reported to be mediated by the Crk nSH3 domain (Gelkop et al., 2001; Sattler et al., 1997), the same SH3 domain that we have described to mediate the Crk-NS1 interaction. To study whether these three proteins are in the same complex, we performed immunoprecipitation of endogenous CrkL from cells transfected with A/Mallard NS1-wt, NS1-AxxA, or NS1-Y89F. In agreement with our earlier results, the A/Mallard NS1 protein readily co-precipitated with CrkL when the SH3 binding motif was intact (NS1-wt and NS1-Y89F), but binding was abrogated when the SH3 binding site was mutated (NS1-AxxA) (see fig. 2B in **II**). p85β was seen to co-precipitate with CrkL when NS1 was not expressed. The expression of NS1-wt or NS1-AxxA did not affect the net association of p85β with CrkL. However, transfection of NS1-Y89F potently inhibited the association of p85β with CrkL. Since the binding of p85β to Crk is mediated by the nSH3 domain of Crk, we hypothesized that NS1 could displace p85β from binding to the nSH3 domain of Crk proteins.

To demonstrate that NS1 could disrupt the preformed p85β-CrkL complex, we co-transfected cells with p85β together with CrkL. The lysates were mixed with increasing amounts of recombinant GST-NS1-Y89F of A/Mallard, or NS1-AxxA as a control. We examined the amounts of p85β that co-precipitated with CrkL by Western blotting. Recombinant NS1-Y89F was able to displace p85β from its complex with CrkL in a dose-dependent manner (see fig. 2C in **II**). To further prove that NS1 is able to displace p85β from a preformed complex with CrkL, we used a mutant construct of p85β (V573M), which prevents the NS1-p85β interaction, and recombinant A/Mallard NS1-wt protein in a similar experiment as above. We obtained a similar result, where NS1-wt could displace p85β-V573M from CrkL in a dose-dependent manner (see supplementary fig. 1 in **II**). From these results and the already known interactions of these proteins (see fig. 3A in **II**), we hypothesized that two alternative trimeric complexes could be formed (see fig. 3B in **II**). The interaction between CrkL and p85β may be indirectly mediated via the dual capacity of NS1 to bind both p85β and CrkL when SH3 binding-competent NS1 is expressed (Complex 1). On the other hand, we also hypothesized, that SH3 binding

deficient NS1 could form a complex with CrkL through association with p85β (Complex 2). The modest affinity of CrkL nSH3 to p85β and/or low abundance of p85β compared to CrkL and NS1 could explain why we cannot detect the association of SH3 binding deficient NS1 with CrkL.

To test our hypothesis, we co-transfected increasing amounts of p85β together with a constant amount of A/Mallard NS1-wt or NS1-AxxA. Co-precipitation of NS1-wt with CrkL was already detectable in the absence of p85β transfection, and the overexpression of p85β did not affect the association (see fig. 4A in **II**). In contrast, increasing amounts of p85β enabled the CrkL-NS1-AxxA co-precipitation in a dose-dependent manner. Likewise, co-precipitation of naturally SH3 binding-incompetent NS1 proteins, A/Udorn and A/WSN, with CrkL was evident when p85β was overexpressed (see fig. 4B in **II**). In strong support for our hypothesis of two alternative complexes, the ability of CrkL to precipitate NS1 proteins of A/Udorn and A/WSN through p85β was abrogated by the p85β-(P294A, P297A) mutation, which impairs the Crk-p85β interaction. Thus, CrkL-p85β-NS1 complex (Complex 2) may exist when an SH3 binding-incompetent NS1 is expressed and it is mediated through the p85β interaction with Crk-SH3 domain. However, assembly of Complex 2, where p85β is bridging the interaction between CrkL and SH3 binding deficient NS1, could only be seen when p85β was overexpressed. Thus, the recruitment of CrkL to p85β-NS1 complexes via p85β seems to be less efficient than recruitment via an SH3 domain mediated CrkL-NS1 interaction.

To further study the model of two alternative trimeric complexes, we made an experiment where A/Mallard NS1-wt, -AxxA or -Y89F were co-transfected with p85β wt, or its mutantsP294A/ P297A, and V573M in all relevant combinations.

To further study the model of two alternative trimeric complexes, we made an experiment where A/Mallard NS1-wt, -AxxA or -Y89F were co-transfected with p85β wt, or its mutants P294A, P297A and V573M in all relevant combinations. The p85β-wt could be co-precipitated with CrkL from cells co-transfected with NS1-wt and NS1-AxxA, whereas expression of NS1-Y89F inhibited this association, which was in line with the earlier results (see fig. 5, panel II, lanes 3-5 in **II**). p85β-V573M, the NS1-binding deficient mutant, had no effect on binding to CrkL but reduced the p85β-CrkL association in cells transfected with NS1-wt and NS1-Y89F (lanes 10 and 13). The expression of p85β-(P294A, P297A), the SH3 binding site mutant, abolished the p85β-CrkL binding (lane 6). This association could be rescued by expression of NS1-wt (lane 7) but not of NS1-AxxA (lane 8) or NS1-Y89F (lane 9). In addition, NS1-wt increased the association of p85β and CrkL (see fig. 5, panel VI, lanes 3 and 7 in **II**). These results confirm that NS1 can serve as a bridging factor between p85β and CrkL in Complex 1 (see fig. 3B in **II**).

To confirm that the trimeric complex of NS1, p85β and CrkL can assemble in the absence of additional cellular factors, we expressed and purified full-length p85β-wt, p85β-(P294A, P297A), NS1-wt and NS1-AxxA proteins, as well as the nSH3 domain fragment of CrkL. The recombinant proteins were mixed in different combinations and proteins associated with p85β were precipitated. p85β-(P294A, P297A) could associate with CrkL-nSH3 only when NS1-wt was present, attesting that Complex 1 could be assembled *in vitro* from purified recombinant proteins (see supplementary fig. 2A in **II**). The SH3 binding-deficient NS1-AxxA could associate with CrkL-nSH3 only when p85β-wt was present, resulting in the assembly of Complex 2. p85β with the SH3 binding site mutation (P294A, P297A) could not mediate the formation of the trimeric complex.

4.1.4 Functional consequences of the trimeric complex (II)

Based on results concerning the rearrangement of the p85β-CrkL complex by NS1, we reasoned that the lower PI3K induction by SH3 binding deficient NS1 proteins could be rescued by overexpression of Crk proteins. To test the idea, we transfected A/Mallard NS1-wt, NS1-AxxA and A/Udorn NS1 with or without CrkL. Overexpression of CrkL had a modest enhancing effect on NS1-wt induced Akt phosphorylation (see fig. 6A and B in **II**), while the weaker PI3K activation by NS1-AxxA was significantly enhanced by CrkL overexpression. A similar potentiation of PI3K activation by A/Udorn NS1 was observed when CrkL was overexpressed.

The SH3 binding function of NS1 has been reported to regulate the activity of c-Abl (Hrincius et al., 2014) Moreover c-Abl phosphorylates p85β (Sattler et al., 1996). We tested whether c-Abl has a role in regulation of PI3K activation by SH3 binding competent NS1 proteins. The inhibition of c-Abl with a potent c-Abl inhibitor, imatinib, suppressed the tyrosine phosphorylation of CrkL but did not have an effect on NS1-induced Akt phosphorylation (see supplementary fig. 3 in **II**). Thus, c-Abl does not seem to have a critical role in this regulation.

4.2 Localization of the Crk-NS1 complex (III)

4.2.1 NS1 mediates nuclear translocation of Crk proteins (III)

To study the localization of NS1 and Crk proteins in IAV infected cells, we analyzed cells infected with recombinant IAVs (see Table 3) by immunostaining and confocal imaging. A/Mallard NS1-wt and NS1-K217E proteins were predominantly seen to localize in the nucleus of infected cells (see fig. 2A in **III**). In mock-infected cells, CrkL was mainly localized in the cytoplasm, while in A/Mallard NS1-wt expressing cells CrkL signal was observed to co-localize with NS1 signal in the nucleus. In contrast, the localization of CrkL in A/Mallard NS1-K217E (SH3 binding deficient mutation) expressing cells did not differ from mock-infected cells. Statistical analysis of CrkL nuclear signal confirmed that the distribution of Crk proteins was strikingly different in A/WSN-NS1$^{Mallard(wt)}$ –infected cells compared to A/WSN-NS1$^{Mallard(K217E)}$ –infected or mock-infected cells (see fig. 2B in **III**). To confirm the translocation of Crk proteins seen with imaging studies, we prepared cytoplasmic and nuclear fractions of cells infected with recombinant viruses. Strong signals of CrkI, CrkII and CrkL proteins could be detected from the cytoplasmic fractions (C) of mock infected cells (see fig. 3A and B in **III**), whereas nuclear fractions (N) did not contain any detectable amounts of these proteins. The distribution of Crk proteins in cells infected with viruses expressing an SH3 binding deficient NS1 protein (NS1$^{Mallard(K217E)}$ or NS1$^{WSN(wt)}$) was identical to mock-infected cells. Remarkably, in cells infected with viruses that contain an SH3 binding competent NS1 (NS1$^{Mallard(wt)}$ or NS1$^{WSN(T215P)}$) all Crk proteins were found in the nuclear fractions, especially CrkL. Thus, the SH3 binding motif of NS1 mediates the translocation of Crk proteins into the nucleus in IAV infected cells.

4.2.2 Functional role of Crk translocation into the nucleus by NS1 (III)

To study whether the NS1-SH3 domain binding mediated translocation of Crk proteins into the nucleus is responsible for enhancing the NS1-induced PI3K activation, we constructed an A/Mallard NS1 protein, which remains predominantly in the cytoplasm (NS1-Cyto). CrkL was observed to co-localize with NS1-Cyto in the cytoplasm in

transfected cells (see fig. 4A in **III**). However, the NS1-Cyto was able to induce the phosphorylation of Akt in similar level as NS1-wt (see fig. 4B in **III**). Thus, the NS1-mediated translocation of Crk proteins is not involved in the activation of PI3K.

Since Crk proteins are involved in the regulation of many tyrosine kinases, we next investigated, whether the nuclear translocation of these proteins by NS1 would have an effect on tyrosine phosphorylation of cellular proteins. Cells infected with recombinant viruses were subjected to cytoplasmic and nuclear fractionation. A new, approximately 135 kDa tyrosine phosphorylated protein could be observed from the nuclear fractions of cells infected with viruses having SH3 binding competent NS1 ($NS1^{Mallard(wt)}$ or $NS1^{WSN(T215P)}$) (see fig. 5 in **III**). This phosphotyrosine-containing protein was not detectable from the nuclear fractions of cells infected with viruses containing SH3 binding deficient NS1 protein ($NS1^{Mallard(K217E)}$ or $NS1^{WSN(wt)}$) or mock-infected cells. Thus, we concluded that the nuclear translocation of Crk proteins by NS1 results in novel tyrosine phosphorylation of a nuclear protein.

5 DISCUSSION

The NS1 protein of IAV is the key factor regulating the host cell responses to facilitate efficient virus replication. It is also considered to be a major virulence factor in IAV infected cells. It inhibits the host cell IFN and antiviral responses by blocking the activation of RIG-I and preventing the processing of cellular mRNA. NS1 also inhibits specific antiviral effector proteins such as OAS and PKR, and modulates apoptosis. In addition, the binding of NS1 to p85β regulatory subunit of PI3K and the subsequent activation of PI3K/Akt pathway is conserved among NS1 proteins of diverse IAV strains (Ehrhardt and Ludwig, 2009; Hale and Randall, 2007). The IAV NS1 protein has been reported to have numerous functions and host cell interaction partners in the infected cell (Ayllon and Garcia-Sastre, 2015). Despite the wealth of such previous data on NS1 and its cellular partners, the exact mechanism by which NS1 acts as an IAV virulence factor, and especially NS1 functions beyond its role as an antagonist of interferon signaling, have remained elusive. In studies reported in this thesis we investigated novel host cell proteins that could be involved in NS1 mediated pathogenesis of different IAV strains.

5.1 Crk-NS1 interaction and PI3K activation

Many viral proteins have evolved an SH3 binding motif in order to exploit host SH3 domain containing proteins to support virus growth and replication. In this thesis, we focused on IAV-host cell interactions mediated by SH3 domains. The sequence analysis of NS1 proteins of different IAV strains revealed that the NS1 protein of Spanish Flu and many avian IAV isolates contain an SH3 binding motif, which was not found in most of the seasonal human IAV strains. To identify new host SH3 interaction partners for the NS1 protein, we used a phage display library that contains virtually all human SH3 domain sequences. We were able to show that this motif mediates avid and specific binding to the nSH3 domain of closely related Crk family adaptor proteins. We also demonstrated that the Crk-SH3 binding motif provided the NS1 proteins with enhanced capacity to activate the PI3K/Akt signaling compared to the NS1 proteins which lack the SH3 binding site. The Crk proteins have been reported to interact with the p85 regulatory subunit of PI3K and to be involved in the activation of the pathway (Akagi et al., 2000; Gelkop et al., 2001; Sattler et al., 1996). Since both p85β and NS1 bind to the same nSH3 domain of Crk proteins, we further sought to examine the biochemical mechanism responsible for the superactivation of PI3K/Akt signaling by SH3 binding competent NS1 proteins. We demonstrated that NS1 proteins with SH3 binding capacity can disrupt naturally occurring p85-Crk interaction, by competing with p85 for binding to the Crk nSH3 domain. This was shown to result in rearrangement of the Crk-p85β complex and in the assembly of a novel trimeric complex of Crk-NS1-p85β (Complex 1) where NS1 is the bridging factor. We also noticed that when SH3 binding incompetent NS1 is expressed, an alternative trimeric complex is formed, where instead of NS1, the p85β serves as the bridging factor by binding to both NS1 and nSH3 domain of Crk. This leads to the formation of NS1-p85β-Crk complex (Complex 2). Because subcellular localization has been shown to be important for the function of both NS1 and Crk (Ayllon and Garcia-Sastre, 2015; Fish et al., 1999; Smith et al., 2002), we also wanted to study the distribution of the Crk-NS1 complex in the host cell. We demonstrated that the SH3 binding motif containing NS1 proteins were able to induce a robust translocation of Crk

proteins from the cytoplasm to the nucleus where the Crk proteins co-localized with NS1 protein in IAV infected cells. The nuclear localization of Crk proteins could not be observed when cells were infected with virus that contains an NS1 protein that lacks the SH3 binding motif. We further showed that this relocalization of Crk by NS1 did not mediate activation of PI3K/Akt by NS1. Instead, we demonstrated that the translocation of Crk into nucleus by NS1 protein was associated with a dramatic change in the tyrosine phosphorylation pattern of nuclear proteins.

The activation of PI3K/Akt pathway by NS1 protein has been reported to be beneficial for the replication and virulence of several IAV strains (Ayllon et al., 2012a; Hale et al., 2006; Hrincius et al., 2012; Shin et al., 2007a). In study **I** we reported that NS1 proteins of Spanish Flu and many avian origin IAV strains has evolved with an enhanced capacity to activate the PI3K/Akt pathway. This potentiation was shown to be dependent on the SH3 binding motif found in these proteins. Although the NS1-induced activation of PI3K/Akt signaling is conserved among different IAV strains, the requirement of the induction for the virus replication and pathogenesis has been shown to be virus strain dependent (Ayllon et al., 2012b). Since the SH3 binding motif is highly conserved in especially avian IAV strains, and only seldom found in human isolated IAV strains, the ability to activate the PI3K pathway more effectively must provide the avian viruses with direct replicative advantage in avian host. Even though many human IAV strains require the NS1 induced PI3K activation for efficient virus replication, providing the viruses with SH3 binding competent NS1 protein does not have positive (or negative) effect on viral replication or virulence (Hale et al., 2010b; Hsiang et al., 2012). However, some exceptions also exist. The human isolated A/PR8 IAV strain was shown to be more pathogenic in mice when the SH3 binding motif was introduced to its NS1 protein (Hrincius et al., 2015).

5.2 Crk-NS1-PI3K complex formation

The NS1-mediated activation of the PI3K pathway has been reported to require a direct association of NS1 with the p85β regulatory subunit of PI3K (Hale et al., 2006; Shin et al., 2007b). The results by Shin et al. indicated that NS1-p85 interaction is mediated by the SH3 domain of p85 (Shin et al., 2007a). However, in study **I**, we were not able to observe any measurable association between SH3 domains of p85α or p85β and NS1. The proposed SH3 binding motif located around amino acid 164, different from the Crk-binding site, was later demonstrated to be important for binding to p85 but not through SH3 domain (Hale et al., 2010a). Earlier studies have also linked the Crk proteins to the activation of PI3K signaling by binding to a proline-rich motif in p85 regulatory subunit of PI3K via their nSH3 domain (Akagi et al., 2002; Akagi et al., 2000; Gelkop et al., 2001; Sattler et al., 1997). Our results in study **II** confirmed that Crk proteins are physically coupled to the activation of PI3K/Akt pathway. We described that the potentiation of pathway activation by SH3 binding competent NS1 proteins is caused by their ability to recruit Crk adaptor proteins to the PI3K complex more efficiently than the SH3 binding incompetent NS1 proteins. The SH3 binding motif in NS1 directs the assembly of a novel trimeric complex (p85β-NS1-Crk; Complex 1), where NS1 connects the p85β and Crk. Interestingly, we could also demonstrate that Crk proteins are involved in PI3K activation also by NS1 proteins that lack the SH3 binding site. In this case, another trimeric complex of NS1, Crk, and p85β-Crk formed, where instead of NS1, the p85β serves as a bridging factor by binding to both NS1 and Crk (NS1-p85β-Crk; Complex 2). Yet, we could only

observe the Complex 2 when p85β was overexpressed, which led us to speculate that the weaker activation of PI3K/Akt observed by SH3 binding incompetent NS1 proteins might be because of the low cellular levels of p85β and lower affinity of p85β to the Crk nSH3 domain. Indeed, we could demonstrate that overexpression of Crk proteins could compensate the lower PI3K/Akt induction seen by the SH3 binding incompetent NS1 proteins compared to SH3 binding competent NS1. Thus, indicating that the SH3 binding competent NS1 provides more efficient recruitment of Crk to the PI3K leading to the formation of Complex 1 and enhanced activation of the pathway. However, the NS1-mediated PI3K/Akt activation was seen to be solely depended on the interaction of NS1 with p85β, which was in line with earlier studies by others (Hale et al., 2006; Shin et al., 2007b), and the association of NS1 with Crk-SH3 domain was able to only enhance the capacity of NS1 to induce the pathway.

Structural studies on NS1-PI3K complex have revealed the mechanism of NS1-induced activation of PI3K, but the precise role of Crk proteins in this requires further studies. Binding of NS1 to the p85β breaks up the inhibitory contacts between the p110 and p85β (Hale et al., 2010a). A possible scenario is that Crk proteins or some Crk associated protein directly contributes to the activation of the complex or is involved in dissociation of the negative regulation between p110 and p85β. Studies by others have reported that the Crk SH2 domain mediates the activation of PI3K by binding to Cbl and activating the Src kinases (Akagi et al., 2002; Song et al., 2010). Thus, the role of Crk in NS1-induced PI3K signaling could be to recruit Src kinases to the NS1-PI3K complex through binding to the Cbl. Since several alternative p85 and p110 isoforms contribute to the formation of PI3K signaling complex depending on the initial stimulus (Vanhaesebroeck et al., 2010), another possibility for the role of Crk in NS1-mediated PI3K activation could be that Crk anchors the PI3K-NS1 complex to a subcellular compartment that is beneficial for the pathway activation by NS1. Yet, we could only observe the Complex 2 when p85β was overexpressed, which led us to speculate that the weaker activation of PI3K/Akt observed by SH3 binding incompetent NS1 proteins might be because of the low cellular levels of p85β and lower affinity of p85β to the Crk nSH3 domain. Indeed, we could demonstrate that overexpression of Crk proteins could compensate the lower PI3K/Akt induction seen by the SH3 binding incompetent NS1 proteins compared to SH3 binding competent NS1. Thus, indicating that the SH3 binding competent NS1 provides more efficient recruitment of Crk to the PI3K leading to the formation of Complex 1 and enhanced activation of the pathway. However, the NS1-mediated PI3K/Akt activation was seen to be solely depended on the interaction of NS1 with p85β, which was in line with earlier studies by others (Hale et al., 2006; Shin et al., 2007b), and the association of NS1 with Crk-SH3 domain was able to only potentiate the capacity of NS1 to induce the pathway.

5.3 Nuclear localization of Crk-NS1 complex

Since the replication of IAV occurs in the nucleus of the host cell, manipulation of nuclear environment during the infection is important for promoting virus propagation. Both NS1 and Crk proteins have described to carry out distinct functions in the nucleus. The NLS sequences efficiently target the NS1 protein into the nucleus, where it, for example, inhibits host gene expression by binding to CPSF30. Crk proteins have also been described with different nuclear functions but since they lack any NLS, their transport from the cytoplasm to the nucleus is regulated by their interaction partners. In study **III**,

we reported a robust nuclear translocation of Crk proteins by SH3 binding competent NS1 which was further associated with tyrosine phosphorylation of an approximately 135 kDa nuclear protein. The size of the protein correlates with the cytoplasmic tyrosine phosphorylated p130Cas, an adaptor protein that binds to the Crk SH2-domain (Birge et al., 1992; Sakai et al., 1994). Another tyrosine phosphorylated Crk-interaction partner that also correlates with the size of the unknown tyrosine phosphorylated protein in the nuclear fraction is c-Abl. Crk associates with c-Abl through the nSH3 domain and the interaction causes the autophosphorylation and activation of c-Abl (Brasher and Van Etten, 2000; Shishido et al., 2001). So far we have not been able to show that either one of these proteins would be a part of Crk-NS1 complex in the nucleus. Since the association of Crk with c-Abl is mediated by the nSH3 domain, the Crk-NS1 interaction would have to be disrupted before the Crk-c-Abl interaction could happen. Additionally, the association of c-Abl with Crk could happen indirectly since NS1 has also been reported to interact with c-Abl (Hrincius et al., 2014). Although this remains an interesting scenario, the interaction of NS1 with c-Abl was linked to inhibition of the c-Abl, not activation. The tyrosine phosphorylated protein observed in the nuclear fraction could also be a protein that is not physically linked to Crk. As the Crk proteins are involved in the regulation of a large number of different protein kinases both in the cytoplasm and the nucleus, the translocation of Crk into nucleus by NS1 may initiate a signaling cascade of different kinases and the tyrosine phosphorylated protein we observe could be acting somewhere downstream of Crk. Further work is needed to reveal the identity of the tyrosine phosphorylated protein and its role in IAV infection.

Previous studies have described different nuclear functions for Crk proteins. It has been shown that in response to type I IFNs and various cytokines, CrkL binds to phosphorylated Stat5 via the CrkL SH2 domain (Fish et al., 1999; Grumbach et al., 2001; Lekmine et al., 2002; Rhodes et al., 2000; Uddin et al., 2003). The resulting CrkL-Stat5 complex translocates into the nucleus where it binds to Stat5-responsive elements to induce the expression of IFN stimulated genes. Thus, the NS1-mediated translocation of CrkL to the nucleus we reported in study **III** could provide another layer of the inhibition of IFN stimulated genes already described for NS1 protein. Importantly, transportation of CrkII into the nucleus has been reported to be pro-apoptotic by activating caspases and binding to the cell cycle regulator Wee1 through the CrkII SH2 domain (Smith et al., 2000; Smith et al., 2002). Apoptosis and the activation of caspases is important for efficient nuclear exit of IAV vRNP complexes (Muhlbauer et al., 2015; Wurzer et al., 2003). Thus, another interesting scenario for NS1-mediated Crk nuclear translocation would be to induce apoptosis in order to promote the exit of vRNP from the nucleus. Underneath the nuclear envelope lies a protein meshwork called the nuclear lamina, composed of nuclear lamins, which is thought to be a common target especially for nuclear replicating viruses. Our unpublished data indicates that viruses expressing SH3 binding competent NS1 may induce the cleavage and dispersion of nuclear lamins compared to viruses in which NS1 lacks the Crk binding capacity. Targeting lamins could be a possible mechanism for viruses bearing Crk binding competent NS1 to promote viral replication.

5.4 Multiple functions of Crk-NS1 interaction

Crk proteins regulate a large set of cellular signaling events. Similarly, NS1 has multiple functions and numerous interaction partners in the host cell. Considering this, it is not a

surprise that Crk-NS1 interaction has been reported to be involved in several cell functions as well. In addition to our observations that Crk-NS1 interaction is involved in the superactivation of PI3K and in manipulating the intracellular distribution of Crk proteins, which leads to changes in tyrosine phosphorylation of nuclear proteins, others have noticed additional functions for this interaction, namely the suppression of c-Abl and JNK signaling (Hrincius et al., 2014; Hrincius et al., 2010). How is the Crk-NS1 complex able to conduct all these functions in infected cell? NS1 protein is expressed in considerable amounts in infected cells and the expression level of Crk proteins is also high. Thus, Crk-NS1 complex would probably be able to participate in different signaling routes at the same time and either one of the proteins could be the limiting factor. NS1 protein has been speculated to be temporally regulated in order to execute all its different tasks at the right time, thus all the distinct functions described for Crk-NS1 complex could also be coordinated to happen in different time points of infection. In addition, one has to bear in mind the differences in the signaling characteristics of the three Crk family members. Hrincius et al. reported that CrkI was the main family member participating in the inhibition of JNK pathway and apoptosis (Hrincius et al., 2010). We were not able to see any variation between the different Crk proteins in the ability to enhance the NS1-induced PI3K activation in study **II**. When we studied the localization of the Crk-NS1 complex in study **III**, we could observe more pronounced translocation of CrkL than CrkI or CrkII into nucleus, but the possible differences of these proteins for the tyrosine phosphorylation of the nuclear protein observed or other possible nuclear functions remain to be studied.

5.5 Diversity in the SH3 binding motif of NS1

The NS1 sequence at the SH3 binding motif (amino acids 212-217) is diverse among different IAV strains. While most avian IAV NS1 proteins contain the Crk-SH3 binding motif, only few human IAV NS1 proteins contain the motif. The critical amino acids, that we reported to be important for the SH3-mediated binding of NS1 to Crk in study **I** (P212, P215, and K217) provides the major variation sites observed for NS1 proteins of different IAV strains. Sequence alignment of multiple NS1 proteins from avian IAV isolates until this date reveals a consensus sequence of ^{212}PPLPPK^{217} (NCBI, 2016), showing that the SH3 binding site is highly conserved in NS1 proteins avian IAVs. In contrast, sequence alignment of human IAV isolate NS1 proteins reveals that these NS1 proteins contain a consensus sequence of ^{212}PPLTPK^{217} (virus isolates from years 2009-2016). Thus, most of the avian influenza A viruses have a proline at amino acid 215, whereas human IAV strains contain a threonine in this position but variations at different positions also occur. Avian IAV subtype H5N1 offers an exception to this rule, since the consensus for NS1 in these viruses is ^{212}LPLPPN^{217} (virus isolates from years 2009-2016), where two amino acids important for the binding have been substituted with other residues (P212L and K217N). Interestingly, the NS1 protein of famous human pandemic IAV strain, the 1918 Spanish Flu, possessed the SH3 domain binding site, but the motif was lost during further adaptation of the virus to human.

5.6 Significance of the NS1 SH3 binding motif for IAV

The NS1 gene of most seasonal IAV strains would need just a single nucleotide change to provide the NS1 protein with the capacity to bind Crk SH3 domain. If it would be beneficial for viral replication and virulence, it could be expected to happen quickly.

Instead, the requirement of the Crk-binding motif in NS1 protein for IAV replication and pathogenesis seems to be virus strain specific. For example, a single nucleotide change in the NS1 sequence of the 2009 pandemic Swine Flu (A/California/04/09) created a functional Crk-binding motif (E217K), but did not enhance virus replication in human or swine cells (Hale et al., 2010b). The virulence or the transmission of the virus was also not affected. Likewise, mutation of T215 to proline to create an SH3 binding site in human IAVs of A/Udorn and the Swine Flu strains did not affect virus replication (Hsiang et al., 2012). Similarly the avian H5N1 subtype IAV, in which NS1 naturally lacks the Crk-binding site, did not benefit from the introduction of the SH3 binding motif into its NS1 protein (Hrincius et al., 2014). In contrast, avian IAV strain that expresses SH3 binding competent NS1 replicated less efficiently when the motif was mutated. On the other hand, human IAV virus, A/PR8 was more pathogenic in mice, when SH3 binding site was introduced in NS1 (Hrincius et al., 2015). Thus, the requirement for the Crk binding site in NS1 is strikingly virus strain dependent, and the viruses naturally lacking the site do not (with some notable exceptions) benefit from the addition of this site. The reason why most avian IAVs seem to benefit from the ability to modulate Crk signaling in the host cell and why it is lost when the virus is transmitted to human host may lie in the natural environment these viruses encounter. The major replication site for avian IAVs in their natural host is the intestine tract, whereas human IAVs infect mainly the trachea. The differences in protein expression and in the temperature of the environment may dictate the necessity of the Crk binding property of NS1 protein for the virus. The Crk proteins are ubiquitously expressed in different human tissues and they are well conserved between different species. However, the expression levels of these proteins in the avian tissues is not known and should be studied. Remarkably, in study **I** we showed that the NS1 protein of the IAV strain that caused the most severe pandemic known of all time, the highly pathogenic Spanish Flu, contain a functional SH3 binding motif. The Spanish Flu IAV has been reported to be extraordinarily virulent and possess higher replication capacity in cell culture and mice than the reference IAVs (Kash et al., 2006; Tumpey et al., 2005). Moreover, the Spanish Flu NS1 has been reported to be more potent in regulating gene expression in host cell compared to NS1 proteins of other IAV strains (Geiss et al., 2002). The SH3 binding site that provides the Spanish Flu NS1 protein with capacity to exploit the host cell signaling machinery by binding to Crk proteins may have contributed to the very highly pathogenic nature the virus had. The adaptation of the virus to contain the SH3 binding site might provide the virus with increased replicative advantage or pathogenic potential also in human cells, which provides a potential threat in novel zoonotic pandemic viruses.

6 CONCLUSIONS

In this thesis, we found a novel, functional SH3 binding motif in the NS1 protein of Spanish Flu and most avian isolated IAV strains but not in NS1 proteins of most human IAV strains. The site was shown to mediate interaction with the nSH3 domain of the Crk-family adaptor proteins. This association provides NS1 protein with enhanced capacity to induce PI3K/Akt signaling. We also described the molecular mechanism behind the PI3K superactivation by SH3 binding competent NS1 proteins. The potentiation of the pathway was shown to be a result of a reorganization of the natural PI3K-Crk complex and the formation of a novel trimeric complex of PI3K, NS1 and Crk. Moreover, we found that Crk proteins also have a general role in NS1-mediated activation of PI3K that is independent from the SH3 binding capacity of NS1. In addition, we described another function for Crk-NS1 interaction. The binding of Crk to NS1 was shown to lead to a robust translocation of cytoplasmic Crk proteins into the nucleus. This function was independent from the induction of the PI3K pathway. Instead, the potent translocation of Crk proteins into the nucleus was linked to tyrosine phosphorylation of a nuclear protein.

The results presented here describe two new mechanisms for IAV to regulate host cell signaling by exploiting host cell SH3 domains. While the capability to activate PI3K signaling by NS1 protein is conserved among most IAV strains, our studies revealed that the SH3 binding competent NS1 proteins are exceptionally potent PI3K activators. In addition, the SH3 binding site enables the NS1 proteins to drag the Crk proteins into the nucleus and consequently manipulate the nuclear environment. Importantly, the presented data also reveal that Crk proteins are important general host cell co-factors of IAV infection, independent from the NS1 Crk SH3 binding motif. The fact that some IAV strains have evolved a more efficient strategy to take maximal use of the Crk proteins to regulate host cell signaling in the cytoplasm and nucleus emphasizes the importance of these proteins as NS1-interactor partners in IAV infection.

Further studies on this subject will provide deeper knowledge on the cell biology of IAV replication. They will provide a new perspective to the IAV strain-specific variation of the SH3-binding capacity of NS1, and hopefully open new avenues for anti-IAV therapeutic development. It would be important to study the relevance of the NS1 SH3 binding motif in avian cells. Detailed characterization of the changes in the nuclear protein phosphorylation pattern is also clearly needed in order to understand better the importance of the relocalization of Crk proteins from the cytoplasm to the nucleus by NS1 for IAV replication and pathogenesis. The role of NS1-Crk interaction for IAV cell biology is undoubtedly important, and deserves special attention also in the future.

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ORIGINAL PUBLICATIONS

I

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Avian and 1918 Spanish Influenza A Virus NS1 Proteins Bind to Crk/CrkL Src Homology 3 Domains to Activate Host Cell Signaling*

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NS1 (nonstructural protein 1) is an important virulence factor of the influenza A virus. We observed that NS1 proteins of the 1918 pandemic virus (A/Brevig Mission/1/18) and many avian influenza A viruses contain a consensus Src homology 3 (SH3) domain-binding motif. Screening of a comprehensive human SH3 phage library revealed the N-terminal SH3 of Crk and CrkL as the preferred binding partners. Studies with recombinant proteins confirmed avid binding of NS1 proteins of the 1918 virus and a representative avian H7N3 strain to Crk/CrkL SH3 but not to other SH3 domains tested, including p85α and p85β. Endogenous CrkL readily co-precipitated NS1 from cells infected with the H7N3 virus. In transfected cells association with CrkL was observed for NS1 of the 1918 and H7N3 viruses but not A/Udorn/72 or A/WSN/33 NS1 lacking this sequence motif. SH3 binding was dispensable for suppression of interferon-induced gene expression by NS1 but was associated with enhanced phosphatidylinositol 3-kinase signaling, as evidenced by increased Akt phosphorylation. Thus, the Spanish Flu virus resembles avian influenza A viruses in its ability to recruit Crk/CrkL to modulate host cell signaling.

Pandemic as well as seasonal outbreaks of influenza A virus represent major threats to global public health. In the last century three major pandemics have occurred, in 1918, 1957, and 1968, caused by H1N1 (Spanish flu), H2N2 (Asian flu), and H3N2 (Hong Kong flu) viruses, respectively. Of these, the Spanish flu was the most severe and is estimated to have caused over 40 million deaths worldwide (1). Recent human infections by highly pathogenic H5N1 avian influenza A viruses have increased the concern that another global pandemic may occur.

Influenza A virus belongs to the *Orthomyxoviridae* family of enveloped viruses. Its genome is organized into eight single-stranded, negative-sense RNA segments that code for 11 identified viral proteins (2). NS1 (nonstructural protein 1) is encoded by the shortest RNA segment 8. It is expressed early in viral replication cycle, and it is not a component of the virus particle (2). Instead, NS1 is a multifunctional virulence factor that promotes virus replication in the host cell and helps to evade antiviral immunity (3–5). In particular, NS1 uses several mechanisms to prevent suppression of influenza A virus replication by the type I interferon system of the host.

Recent studies have demonstrated that during influenza A virus infection NS1 protein activates the phosphatidylinositol 3-kinase (PI3K)[3] signaling pathway, apparently via its association with the p85 regulatory subunit of PI3K (6–9). Activation of the PI3K pathway seems to be important for influenza A virus replication, because in cell culture studies recombinant viruses with mutations that prevented binding of NS1 to p85 formed much smaller plaques and grew to 10-fold lower titers than the wild-type virus (9). Moreover, compounds that inhibit PI3K can strongly suppress influenza A virus replication (7, 9, 10).

Hale *et al.* (9) showed that the tyrosine residue 89 (Tyr-89) of NS1 protein serves a critical role in mediating binding to p85β. This tyrosine lies in the context similar to a Y*X*NM motif, which upon tyrosine phosphorylation can serve as a high affinity binding site for the SH2 domain of p85 (11), but apparently NS1 interacts with p85β in an SH2-independent manner (12). In addition, p85 contains an SH3 domain, and Zhou and co-workers (6, 13) have suggested that a P*XX*P sequence in NS1, which resembles the consensus of an SH3-binding motif (see below), may also contribute to the p85 interaction.

SH3 domains are small protein modules that mediate inter- and intramolecular protein interactions and are often found in proteins regulating cellular signaling pathways, cytoskeletal organization, and membrane trafficking (14, 15). SH3 domains recognize short proline-rich sequences, which are typically characterized by (+)-*X*ΦP*XX*P (class I) or P*X*ΦP*X*-(+) (class II) consensus sequences (where *X* is any amino acid; (+) indicates a positively charged residue; and Φ indicates a hydrophobic residue) (15–17). Since the discovery that the human immunodeficiency virus type 1 pathogenicity factor Nef regulates the

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[3] The abbreviations used are: SH3, Src homology 3; PI3K, phosphatidylinositol 3-kinase; IFN, interferon; MBP, maltose-binding protein; TBS, Tris-buffered saline; GST, glutathione *S*-transferase; HA, hemagglutinin; ISRE, interferon-stimulated response element; WT, wild type.

host cell via binding to SH3 domains of Src family protein kinases (18), SH3 domain binding capacity has been demonstrated for many other proteins encoded by viral as well as bacterial pathogens (19–24).

We noted that, unlike most other NS1 proteins from human strains of influenza, the NS1 sequence of the 1918 pandemic influenza virus (A/Brevig Mission/1/18/H1N1 (25)) contains a perfect class II consensus SH3-binding sequence, and in this regard it resembles many avian strains of influenza (26).

We have recently generated an essentially complete (n = 296) collection of human SH3 domains in the form of a phage display library to allow comprehensive and unbiased identification of preferred SH3 partners for cellular and viral ligand proteins of interest (27). In this study we have made use of this novel research tool to identify the Crk family adapter proteins as high affinity ligands for the NS1 protein of the 1918 virus.

EXPERIMENTAL PROCEDURES

Cells and Viral Infections—Human embryonic kidney 293FT, human hepatocellular carcinoma Huh-7, and human A549 lung carcinoma cell lines were maintained in Dulbecco's modified Eagle's medium high glucose supplemented with 0.6 μg/ml penicillin, 60 μg/ml streptomycin, 10% fetal bovine serum, and 2 mM glutamine. Influenza A virus strains A/mallard/Netherlands/12/2000 (H7N3) and A/Udorn/72 (H3N2) were grown in 11-day-old embryonated eggs, and the virus stock was aliquoted and stored at −70 °C. The hemagglutination titers of the viruses were 256, and their infectivity in A549 cells was 1×10^7 and 2×10^7 plaque-forming units per ml, respectively. Virus infection of A549 cells was carried out in Dulbecco's modified Eagle's medium supplemented with 2% fetal bovine serum and antibiotics for 20 h at a multiplicity of infection of 5 plaque-forming units/cell.

To metabolically label virus-infected cells with [^{35}S]methionine, the cells were washed with and changed into methionine-free media supplemented with 2% fetal bovine serum and 0.5 mCi of [^{35}S]Met (GE Healthcare) 4 h after infection and grown for an additional 16 h. At 20 h after infection cells were collected, washed twice with cold phosphate-buffered saline, and lysed.

Plasmid Constructs—A synthetic gene fragment encoding A/Brevig Mission/1/18 NS1 was purchased from GENEART (Regensburg, Germany). A/mallard/Netherlands/12/2000/H7N3 cDNA was cloned from total cellular RNA of virus-infected cells by standard methods. A/Udorn/72 and A/WSN/33 NS1 inserts were derived from pcDNA3.1-based constructs already described (28). To generate the mammalian NS1 expression vectors, these fragments were inserted into the EF1α enhancer-driven vector pEBB-mycN by PCR-mediated cloning and confirmed by DNA sequencing. pEBB-mycN is a derivative of pEBB (from Bruce Mayer, University of Connecticut) (29), in which translation starts upstream of the insert to include a Myc epitope-containing peptide (MEQKLISEEDLGS) at the N terminus. Codon changes to A/Brevig and A/Mallard NS1 gene were generated by overlap PCR mutagenesis, cloned into pEBB-mycN, and verified by sequencing. Bacterial GST and MBP fusion protein expression vectors for NS1 proteins were constructed by inserting the corresponding NS1 cDNAs into pGEX-4T1 (GE Healthcare) and pMAL-c2x (New England Biolabs) vectors, respectively. To generate the plasmid p85α-BP, an oligonucleotide duplex encoding for the peptide CLNCFRPLPPLPPPPR (30) was inserted into the polylinker of pMAL-c2x.

A DNA fragment encoding for a 123-amino acid biotin acceptor domain from *Propionibacterium shermanii* transcarboxylase (start, MKLK; end, IKIG) was PCR-amplified from the PinPoint-Xa1 T-vector (Promega) and inserted between the GST gene and the multiple cloning site of pGEX-4T1 to generate pGEX-PP. Codon optimized cDNAs for the SH3 domains of Crk, CrkL, p85α, p85β, and Eps8L1 derived from the human SH3 library (27) were subsequently cloned in-frame after the biotin acceptor domain in pGEX-PP. To generate C-terminally biotinylated Crk and CrkL expression constructs, human CrkII and CrkL cDNAs with stop codons replaced by a KpnI site were fused with the above-described biotin acceptor domain fragment in pEBB.

ISRE-Luc reporter plasmid contains a 30-bp interferon-stimulated response element-containing fragment from the ISG15 gene (31) in front of a minimal thymidine kinase promoter driving firefly luciferase expression (obtained from J. Darnell Jr., Rockefeller University, New York). As a control for transfection efficiency and cell viability, we used the plasmid pcDNA-Renilla, which was created by inserting *Renilla* luciferase cDNA from pRL-null (Promega) into pcDNA3.1/Hygro vector (Invitrogen).

Antibodies and Other Reagents—The following antibodies were used in this study: mouse anti-Myc (Sigma), mouse anti-CrkL (Upstate), mouse anti-phospho-Akt(Ser-473) (Cell Signaling Technology), rabbit anti-NP (32), mouse anti-hemagglutinin (HA, Santa Cruz Biotechnology), and guinea pig anti-NS1 (28). Streptavidin IRDye800CW, IRDye 800CW goat anti-mouse IgG, and IRDye680 goat anti-mouse IgG were from LI-COR Biotechnology. Secondary horseradish peroxidase-conjugated anti-guinea pig antibodies were from Jackson ImmunoResearch.

Recombinant Proteins and Binding Assays—GST and MBP fusion proteins expressed from pGEX-4T1 and pMAL-c2x protein expression vectors were purified using glutathione-Sepharose 4B (GE Healthcare) or amylose resin (New England Biolabs), according to the manufacturer's instructions. Screening of the human SH3 phage library using the GST-NS1 fusion proteins was done as described previously (27). Recombinant protein binding assay was done as in Kärkkäinen *et al.* (27) with some modifications. MBP-NS1 proteins, MBP-p85α-BP, or plain MBP was coated on 96-well plates (200 ng/well). Wells were blocked with 1.5% bovine serum albumin in Tris-buffered saline (TBS) for 1 h and washed twice with TBS + 0.05% Tween 20 (TBST). MBP proteins were then incubated with 2-fold dilutions of GST-biotin-SH3 domains in TBS for 1.5 h. Wells were then washed three times with TBST, followed by a 1-h incubation with streptavidin-biotinylated horseradish peroxidase complex (1:2000 dilution in TBS; GE Healthcare). After three washes with TBST, 50 μl of substrate reagent ABTS Single Solution (Invitrogen) was added, and the absorbance at 405 nm was measured 20 min later.

Protein Pulldowns and Western Blots—For protein pulldown experiments, 293FT cells were transfected by standard calcium phosphate precipitation method with 10 μg of NS1 expression vectors in 10-cm plates. For avidin pulldown experiments cells were transfected also with 3 μg of Crk or CrkL expression constructs encoding for C-terminally biotinylated proteins. 48 h after transfection, cells were lysed on ice with 1% Nonidet P-40 lysis buffer (150 mM NaCl; 50 mM Tris-HCl, pH 7.9; 1% Nonidet P-40). Cell lysates were used for immunoprecipitation with anti-CrkL antibody and Dynabeads protein A magnetic beads (Invitrogen) or avidin pulldowns with Tetralink tetrameric avidin resin (Promega). To examine the phosphorylation status of Akt, Huh-7 cells in 6-well plates were transfected with 4 μg of Myc-tagged NS1 expression plasmids or empty plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. 48 h after transfection cells were lysed on ice with 1% Nonidet P-40 lysis buffer, and lysates were used for Western blotting to detect phospho-Akt or myc-NS1. Immobilon Western chemiluminescent horseradish peroxidase substrate (Millipore) or Odyssey infrared imaging system (LI-COR Biosciences) was used for detection.

Reporter Gene Assays—To measure the activation of the ISRE promoter, Huh-7 cells were transfected in 12-well plates with 0.2 μg of ISRE-Luc, 5 ng of pcDNA-Renilla, and 1 μg of NS1 expression vectors or empty vector using Lipofectamine 2000 (Invitrogen). 22 h post-transfection, the reporter gene was induced by treatment with 100 IU/ml of IFN-β (Betaferon, Schering) for 7 h. Cells were lysed with Passive Lysis Buffer (Promega), and the luciferase activity was measured using dual-luciferase assay system (Promega) and Sirius luminometer (Berthold detection systems). *Renilla* luciferase construct was used as an internal control to normalize relative luciferase activity.

RESULTS

Consensus SH3-binding Motif in NS1—To look for potential virally encoded ligands for cellular SH3 proteins, we used the ScanProsite search engine to identify consensus SH3 domain-binding motifs in viral protein sequences in the Swiss-Prot/TrEMBL data base. One interesting protein that was noted to contain a perfect class II SH3-binding motif was NS1 of the 1918 pandemic influenza A virus (A/Brevig Mission/1/18/H1N1; A/Brevig below). Analysis of NS1 protein sequences from other strains of influenza A revealed that this sequence motif is very common among avian influenza A viruses but only rarely found in viruses isolated from humans (Fig. 1). In addition to A/Brevig, only three other human-derived NS1 sequences containing this motif could be found from the NCBI Influenza Virus Resource data base. Notably, two of these viruses represented recent zoonotic transmissions from birds with an H5N1 virus (A/Hong Kong/481/97/H5N1 (33)) and an H7N3 virus (A/Canada/rv504/2004/H7N3 (26)). Accordingly, this sequence motif is not present in the NS1 proteins of human influenza A viruses commonly used for laboratory studies, such as A/WSN/33 (H1N1), A/PR/8/34 (H1N1), and A/Udorn/72 (H3N2) (see Fig. 1).

Identification of SH3 Partners of NS1—To examine their SH3 binding potential and preferences, NS1 proteins

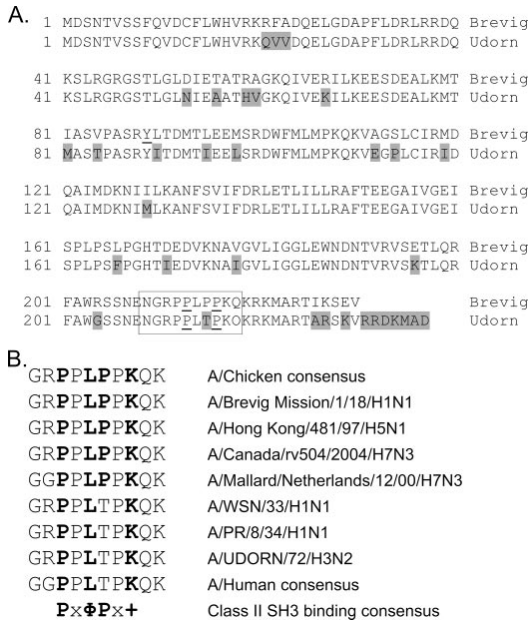

FIGURE 1. **Amino acid sequences of selected influenza A virus NS1 proteins.** *A,* alignment of the complete influenza A virus NS1 sequences of A/Brevig Mission/1/18/H1N1 (Brevig) and A/Udorn/72/H3N2 (Udorn) sequences. Residues in Udorn that differ from the corresponding amino acids in Brevig are highlighted in *gray*. Tyrosine (position 89) and proline (positions 164, 167, 213, and 216) previously implicated in binding to the SH2 and SH3 domains of PI3K-p85 (6, 9) are *underlined*. The region containing a consensus SH3-binding motif in the Brevig sequence is *boxed*. *B,* shown are amino acid sequences within in the *region boxed* in *A* in NS1 proteins from the indicated influenza A viruses, as well as chicken (based on 1996 isolates) and human (based on 2005 isolates) consensus sequences. The sequence *Px*Φ*Px*+ (where *x* denotes any amino acid, Φ indicates a hydrophobic residue, and + indicates an arginine or a lysine) indicates a class II SH3-binding consensus motif (critical amino acids in *boldface*).

encoded by A/Brevig and an avian strain containing the same consensus motif (A/mallard/Netherlands/12/00/H7N3; A/Mallard below) were expressed as GST fusion proteins in *Escherichia coli* and used as ligands for affinity screening of our comprehensive human SH3 phage display library (27). Both proteins bound avidly to SH3 clones in the library as compared with plain GST protein used as a control for nonspecific binding (not shown). Sequencing of the phagemid genomes obtained after a single round of affinity selection with these NS1 proteins revealed that more than 90% of the phages contained the N-terminal SH3 domain of the adapter protein Crk or its close homologue CrkL.

To confirm and study in more detail the NS1/SH3 interactions revealed by phage screening, we generated recombinant GST fusion proteins of the N-terminal SH3 domains of Crk and CrkL. Because the SH3 of p85 has been suggested to bind to P*XX*P sequences found in many influenza A NS1 proteins (6) (see Fig. 1) we also generated GST-SH3 fusion proteins of p85α and p85β. In addition, the SH3 of Eps8L1, which is known to prefer atypical P*XX*DY-containing ligands (34), was included as a negative control. Between the GST and SH3 moieties in these

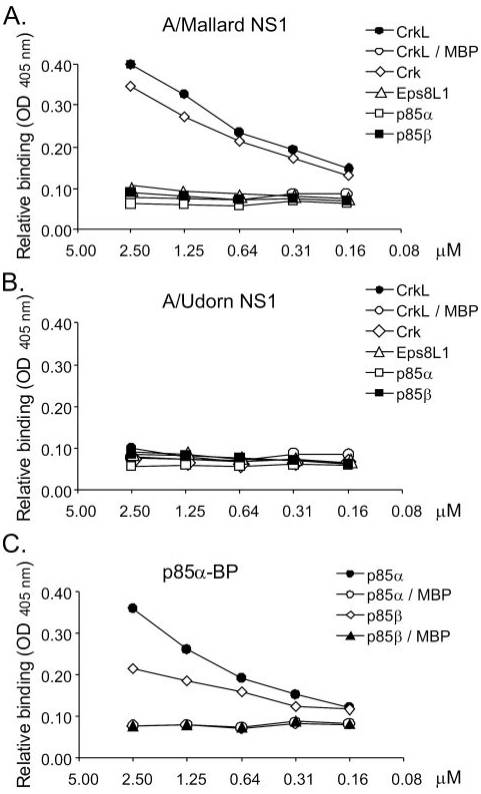

FIGURE 2. **Semi-quantitative analysis of binding of recombinant SH3 domains to NS1 proteins.** A/Mallard (*A*) and A/Udorn (*B*) NS1 proteins were expressed as MBP in *E. coli* and used to coat 96-well plates. These wells were incubated with 2-fold dilutions (ranging from 2.5 to 0.16 μM) of the indicated recombinant SH3 domains expressed as biotinylated GST fusion proteins, followed by detection of binding with an enzymatically labeled streptavidin. As a control for nonspecific binding, additional wells were coated with plain MBP and similarly probed with CrkL SH3 domains (*CrkL/MBP*). In addition, an artificial protein (*p85α-BP*) consisting of MBP fused to a high affinity p85α-SH3 ligand peptide (CLNCFRPLPPLPPPPR) (30) was used as an immobilized target protein to test binding of p85α-SH3 and p85β-SH3. As in *A* and *B*, wells coated with plain MBP (p85α-SH3/MBP and p85β/MBP) were used as controls (*C*).

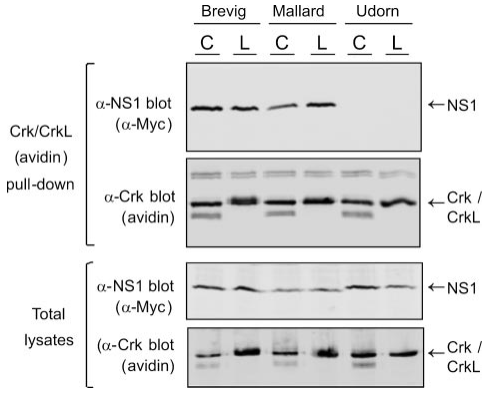

FIGURE 3. **Co-precipitation of A/Brevig, A/Mallard, and A/Udorn NS1 proteins with full-length Crk and CrkL proteins from transfected cells.** Myc-tagged expression vectors for the indicated NS1 proteins were transfected into 293FT cells together with a biotin-acceptor domain-tagged vector for Crk (*C*) or CrkL (*L*). Material precipitated with avidin-coated beads from lysates of the co-transfected cells were analyzed by Western blotting using an anti-Myc antibody or a labeled avidin reagent, as indicated. Aliquots of the lysates were collected before avidin pulldown and subjected directly into Western blot analysis (*two bottom panels*).

fusion proteins, we inserted a biotin acceptor domain from *P. shermanii* transcarboxylase, which during expression in *E. coli* becomes efficiently biotinylated at a single lysine residue, and can be conveniently detected with avidin-based reagents (35). As targets for these SH3 domains, we expressed maltose-binding protein (MBP) fusion constructs A/Udorn, A/Mallard, and A/Brevig NS1 proteins. The latter were coated onto the bottom of 96-well plates and probed with serial dilutions of biotinylated Crk, CrkL, p85α, p85β, or Eps8L1 SH3 domains in an enzyme-linked immunosorbent assay-like sandwich assay.

As shown in Fig. 2, SH3 domains of Crk and CrkL bound avidly to A/Mallard NS1-coated wells, showing significant binding signals even when tested at sub-micromolar concentrations (see Fig. 2*A*). By contrast, p85α and p85β SH3 domains were equally negative in binding to A/Mallard NS1 as was Eps8L1 SH3 or CrkL SH3 in binding to the control wells coated with plain MBP. Although the absolute binding to Crk/CrkL SH3 domains was slightly weaker, very similar results were obtained by using A/Brevig NS1 as the immobilized ligand (data not shown). In agreement with the lack of a consensus SH3-binding motif in A/Udorn NS1, none of the tested SH3 domains showed measurable binding to Udorn NS1 protein (Fig. 2*B*). To confirm the functionality of the p85 SH3 domain proteins used in this study, we fused MBP with a peptide (CLNCFRPLPPLPPPPR) that has been optimized for binding to p85α using phage display (30). Positive binding to this peptide under identical assay conditions (Fig. 2*C*) indicated that the failure of p85α and p85β SH3 domains to bind to the NS1 proteins of A/Mallard and A/Udorn (Fig. 2, *A* and *B*) as well as A/Brevig and A/WSN (data not shown) was indeed a valid result and not because of a general lack of functionality of our p85 SH3 proteins.

Binding of Crk and CrkL to NS1 in Transfected Cells—To extend these findings to full-length proteins expressed in human cells and to compare NS1-binding by Crk and CrkL, we generated expression vectors for these proteins tagged at their N termini with the transcarboxylase biotin acceptor domain. As in our previous studies on divergent protein interactions (36), this strategy allowed sensitive and equal detection of the tagged Crk and CrkL proteins when co-transfected into 293FT cells together with NS1 proteins from different influenza A viruses (Fig. 3). To ensure equal detection of the different NS1 proteins used in this experiment, these expression constructs were tagged with a Myc peptide epitope. Similar expression of all NS1 proteins in the lysates of transfected cells and equal recovery of the biotinylated Crk and CrkL proteins using avidin-coated beads were observed, whereas the amounts of co-precipitated NS1 proteins differed dramatically (Fig. 3, *top panel*). Both Crk and CrkL associated strongly with A/Brevig and

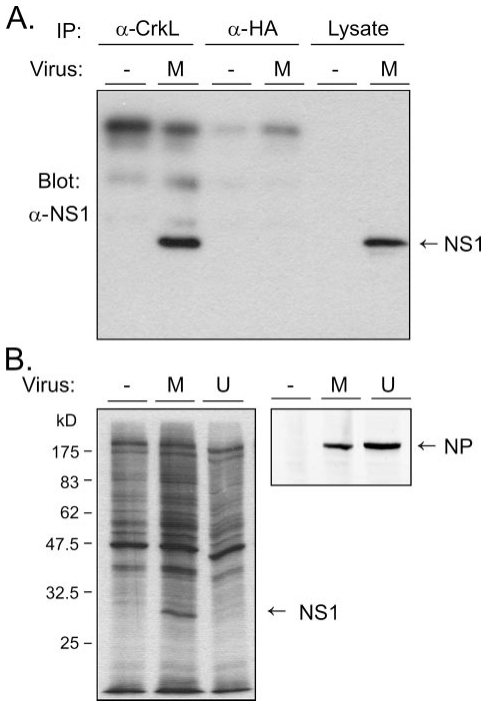

FIGURE 4. **Co-precipitation of NS1 with endogenous CrkL from influenza A virus-infected cells.** *A,* lysates from control A549 cells (−) and cells infected with A/mallard/Netherlands/12/00/H7N3 virus (*M*) were subjected to immunoprecipitation (*IP*) with anti-CrkL (α-*CrkL*) or control (α-*HA*) monoclonal antibodies. These precipitates and aliquots of the corresponding lysates were analyzed by Western blotting using a polyclonal anti-NS1 antiserum. *B,* lysates of [^{35}S]methionine-labeled control A549 cells (−) or similarly labeled cells infected with A/mallard/Netherlands/12/00/H7N3 (*M*) or A/Udorn/72 (*U*) viruses were subjected to immunoprecipitation with an anti-CrkL antibody, followed by SDS-PAGE and autoradiography (*left panel*). The labeled lysates were also subjected to Western blotting with an anti-nucleoprotein (*NP*) antibody (*right panel*).

A/Mallard NS1 proteins, but they failed to co-precipitate any A/Udorn NS1. Binding to A/WSN NS1 was equally negative (data not shown). Thus, in good agreement with the recombinant protein data involving isolated Crk/CrkL SH3 domains, in transfected cells full-length Crk/CrkL proteins bound well to NS1 proteins containing a class II SH3-binding consensus site but not detectably to NS1 proteins lacking this motif.

CrkL-NS1 Complex in Influenza A Virus-infected Cells—Because of the similar NS1 binding profiles of Crk and CrkL, lack of known differences in their cellular functions, and the availability of a good antibody against Crk, NS1 binding by CrkL was chosen as the subject of our subsequent studies. To examine whether binding of endogenous cellular CrkL with NS1 protein produced during influenza A virus infection could be demonstrated, we infected A549 cells with A/Mallard and examined the presence of NS1 in anti-CrkL immunoprecipitates from lysates of these cells. As shown in Fig. 4*A*, a readily detectable amount of NS1 co-precipitated from the A/Mallard-infected cells using the α-CrkL antibody, corresponding in intensity to the NS1 signal derived from a fraction of the total lysate equaling 10% of the fraction used for the α-CrkL immunoprecipitation. By contrast, no such signal was seen in anti-CrkL immunoprecipitates from uninfected cells or from control (α-HA) immunoprecipitates from the A/Mallard-infected cells, thus confirming the specificity of the observed CrkL/NS1 co-precipitation.

A similar A549 infection experiment was carried out in which A/Udorn was included for comparison. Because of the sequence divergence of the A/Mallard and A/Udorn NS1 proteins, quantitative comparison of the expression levels of these proteins in their native form was not possible using any of the immunological reagents that we tested (data not shown). Therefore, we chose to infect A549 cells with matched titers of A/Mallard and A/Udorn viruses, and subjected the infected cells to metabolic labeling with [^{35}S]methionine. Because A/Mallard and A/Udorn NS1 proteins contain a comparable number of methionine residues, their presence in anti-CrkL immunoprecipitates could be accurately compared by autoradiography (Fig. 4*B*).

In good agreement with the Western blotting data (Fig. 4*A*) an NS1-sized protein was co-precipitated by the anti-CrkL antibody from A/Mallard-infected cells but not from uninfected cells (Fig. 4*B*). A faint signal corresponding to a slightly larger protein (as expected for A/Udorn NS1) could also be detected from A/Udorn-infected cells in overexposures of the autoradiogram shown in Fig. 4*B* and other similar experiments (data not shown). Thus, despite its lack of direct CrkL binding capacity (Figs. 2 and 3), in infected cells A/Udorn NS1 might yet be weakly associated with CrkL. Equal infection of the cells with the A/Mallard and A/Udorn viruses was confirmed by Western blotting using an antiserum against nucleoprotein, which shows high amino acid sequence conservation between different influenza A strains (Fig. 4*B*, *right panel*). Together these data confirmed binding of endogenous CrkL to NS1 in virus-infected cells, and underscore the important role of the SH3 binding capacity of NS1 alleles like A/Mallard in promoting this interaction.

Characterization of the Crk/CrkL SH3-binding Site in NS1—To confirm that the class II SH3-binding motif in A/Mallard and A/Brevig NS1 protein was indeed critical for their capacity to bind to the Crk family proteins, we generated a series of point mutated variants of A/Brevig NS1 shown in Fig. 5*A*. Mutant 1 (M1) contained alanine residues in place of both of the P*XX*P-defining proline residues of the class II consensus motif (P212A/P215A). Mutant 2 (M2) contained a similar double alanine substitution but affected the additional P*XX*P sequence that is embedded within but is not part of the consensus SH3-binding motif in A/Brevig NS1 (P213A/P216A). These proline residues are conserved in NS1 proteins of most human and avian influenza viruses, including A/Udorn, and were pointed out by Shin *et al.* (6) as a potential docking site for p85-SH3. Mutants 3 and 4 involved changes in the positively charged residue of the consensus motif. In M3 this charge was reversed by a lysine to glutamic acid substitution (K217E), whereas the lysine to arginine substitution in M4 (K217R) maintained this functionally important positive charge. In fact, arginine is the positively charged residue in most class II SH3-binding sites,

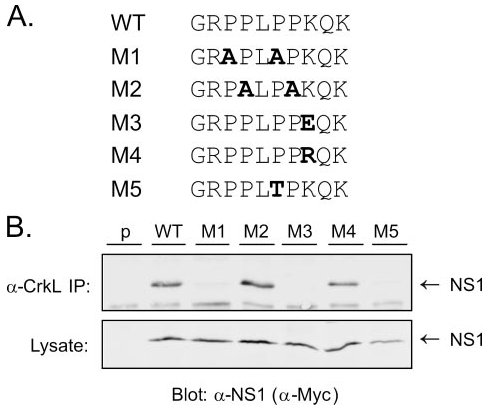

FIGURE 5. **Mutational analysis of NS1 residues involved in binding to cellular CrkL.** *A,* class II SH3-binding motif-containing region of wild-type A/Brevig NS1 protein (WT) and its derivatives (*M1–M5*) carrying the indicated mutations are shown. *B,* Myc-tagged expression vectors for WT or mutated variants of A/Brevig NS1 protein (*M1–M5*) or an empty control vector were transfected into 293FT cells. Lysates of these cells were subjected to anti-CrkL immunoprecipitation (*IP*), and these precipitates along with aliquots of the original lysates were analyzed by Western blotting with an anti-Myc antibody.

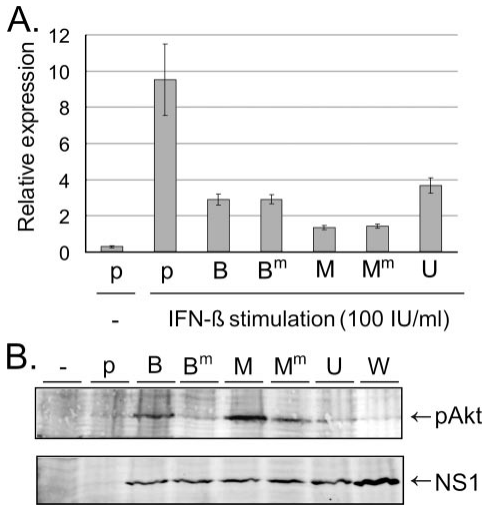

FIGURE 6. **Functional analysis of the role of Crk/CrkL SH3 domain binding capacity in regulation of cellular signaling pathways by NS1.** *A,* Huh-7 cells were transfected with an ISRE-dependent firefly luciferase reporter vector together with an expression vector for A/Brevig (*B*), A/Mallard (*M*), or A/Udorn (*U*) NS1 or SH3 binding-deficient mutants (*M1* in Fig. 5) of A/Brevig (B_m) or A/Mallard (M_m) NS1 proteins, or an empty control plasmid vector (*p*). In addition, a vector stably expressing *Renilla* luciferase was included in all cases to monitor transfection efficiency and cell viability. After 22 h, one culture of cells transfected with the control vector was left untreated, and the other plates were stimulated for 7 h with 100 IU/ml of IFN-β, followed by determination of firefly and *Renilla* luciferase activities of lysates prepared from these cells. ISRE-dependent gene expression is shown as mean values of the ratios of firefly and *Renilla* luciferase activities. *B,* Huh-7 cells were transfected with the NS1 or control expression vectors used in *A* plus a vector for A/WSN/33 NS1 (*W*). 48 h later the cells were collected and analyzed for their PI3K pathway activity by Western blot analysis of their lysates using an antibody specific for the phosphorylated form of Akt. Uniform expression of the NS1 proteins was confirmed by probing the blots with an anti-Myc tag antibody.

but Crk and CrkL SH3 domains have a characteristic property of preferring a lysine residue in this position (37). Mutant 5 (M5) contained a proline to threonine substitution (P215T) affecting the first proline residue of the consensus motif, thus mimicking the NS1 protein sequence in this region of A/Udorn (and practically all human influenza A virus NS1 proteins; see Fig. 1).

The capacity of Myc epitope-tagged NS1 proteins carrying these mutations to bind to endogenous CrkL protein in transfected 293FT cells was studied by a co-immunoprecipitation assay. As shown in Fig. 5*B,* NS1 was equally expressed in all transfected cells, but M1 and M3 that carried class II consensus motif-disrupting mutations could not be co-precipitated with CrkL. The single P215T change (M5) resembling NS1 proteins like that of A/Udorn also abolished binding to CrkL. Instead, association of M2 with CrkL was indistinguishable of unmodified A/Brevig NS1 (WT). In agreement with the published binding preferences of Crk/CrkL SH3 (37), binding of M4 to CrkL was diminished ~50% compared with WT (Fig. 4*B* and data not shown). These data formally establish the critical role of the class II SH3-binding motif found in A/Brevig and many avian strains of influenza A in Crk/CrkL association, and confirm the preference of Crk/CrkL SH3 domains for lysine-containing class II-binding sites.

Role of SH3 Binding in Cellular Functions of NS1—To study the functional role and relevance of Crk/CrkL binding by NS1, we tested the activities of A/Brevig and A/Mallard NS1 proteins and their SH3 binding-deficient derivatives in two different cellular functions that have been reported for NS1, namely inhibition of interferon-induced gene expression and activation of the PI3K signaling pathway. The former apparently involves multiple effector functions of NS1, notably the ability of NS1 to interfere with post-transcriptional mRNA processing (38–40). As already discussed, the latter involves association of NS1 with the p85 subunit of PI3K and can be followed by examining the phosphorylation status of the protein kinase Akt/PKB (6, 8, 41).

When Huh-7 cells were transfected with an ISRE-containing reporter plasmid together with an empty control expression vector (*p*), a robust increase in luciferase activity was observed upon stimulation of these cells with interferon (IFN-β) (compare the *two leftmost bars* in Fig. 6*A*). When a vector expressing NS1 of A/Brevig (B), A/Mallard (M), or A/Udorn (U) was included, the IFN-β-induced increase in reporter gene expression was strongly suppressed. Similar inhibition was also seen when SH3 binding-deficient mutants (corresponding to *M1* in Fig. 5*A*) of A/Brevig (B^m) or A/Mallard (M^m) were tested, which together with the activity of A/Udorn in this assay indicated that binding to Crk/CrkL was not required for the capacity of influenza A NS1 proteins to suppress interferon-induced gene expression.

By contrast, SH3 binding by NS1 was found to correlate with a significant increase in Akt phosphorylation in Huh-7 cells transfected with these vectors. As noted by others (6), cationic lipid-mediated transfection of an empty expression vector plasmid was sufficient to induce a minor activation of PI3K signaling. However, in agreement with earlier reports (6, 8), the use of vectors encoding NS1 of A/Udorn (*U*) or A/WSN (*W*) resulted

in further accumulation of pAkt as an indication of PI3K activation (see Fig. 6*B*). By comparison, the levels of pAkt were significantly higher in cells transfected with A/Brevig (*B*) or A/Mallard (*M*) NS1 constructs (Fig. 6*B* and data not shown). This increased capacity of A/Brevig and Mallard NS1 to activate PI3K signaling was lost when the class II consensus SH3-binding motif of these proteins was disrupted by the M1 mutation (*lanes* B^{m1} and M^{m1} in Fig. 6*B*).

DISCUSSION

Influenza A viruses are highly virulent and can infect a broad range of mammalian and avian species. Several influenza A virus gene products such as HA, viral polymerases, nucleoprotein, and NS1 play a role in the virulence of the virus (42, 43). Although the HA protein largely determines the pathogenicity and species specificity of the virus, NS1 protein has a uniform role in regulating host cell responses during the infection independent of the virus type or the animal species the virus is infecting. NS1 protein is a double strand RNA-binding protein, and it can interfere with the functions of other double strand RNA-binding proteins such as the RIG-I, protein kinase R, and oligoadenylate synthetases (44–46) that regulate the induction and antiviral effects of interferons, respectively. NS1 protein is also targeted into the host cells nucleus (28), where it can interfere with the processing of host cell pre-mRNAs, including those of antiviral mRNAs rendering them susceptible for degradation (47). Influenza A virus also takes advantage of the host cell signaling pathways activated during the infection in such a way that signal transduction involving, for example, NF-κB, mitogen-activated kinase cascades, and the PI3K pathway is altered to optimize virus replication.

In this study we have shown that the NS1 protein of the 1918 pandemic influenza A virus (A/Brevig) contains a functional SH3 interaction motif that mediates avid binding to the N-terminal SH3 domain of the adapter proteins Crk and CrkL. This sequence motif is common in NS1 proteins of avian influenza A viruses, but besides A/Brevig can be found only in three of the 4505 human-derived NS1 protein sequences available in the NCBI influenza A data base. Interestingly, two of these cases represent zoonotic infections of humans with avian H5N1 and H7N3 viruses.

Amino acid variation in the NS1 gene has been shown to correlate with the pathogenicity of H5N1 strains of avian influenza virus in chickens (48), and the NS1 gene of A/Brevig has been found to be more potent than the reference NS1 proteins in regulating host cell gene expression (49). Thus, although human influenza A infection clearly differs from that of avian hosts in that the capacity of NS1 for Crk/CrkL SH3 binding appears to be generally counter-selected in the formed case, adaptation of the virus to exploit this connection to the cellular signaling machinery also in human cells might provide the virus with increased replicative or pathogenic potential. It was recently shown that the 1918 virus shows a higher replication capacity in tissue culture and in animal models (50, 51).

Several different viral species have been found to stimulate PI3K signaling to increase their replication and/or to prevent apoptotic death of the host cell (for a review, see Ref. 52). Indeed, NS1-mediated activation of PI3K signaling was recently shown to enhance influenza A virus replication (9, 10) and limit apoptosis in infected cell cultures (8). Our observation that a functional Crk/CrkL-binding motif provided the NS1 proteins of A/Brevig and A/Mallard with an increased ability to induce PI3K signaling could therefore readily explain a potentially higher replicative capacity of viruses expressing Crk/CrkL binding-competent NS1 proteins.

Crk/CrkL proteins were first identified as cellular counterparts of the protein encoded by the v-crk oncogene of the avian sarcoma virus CT10 (53), and have since been found to be ubiquitously involved in numerous signaling pathways regulating diverse functions in different cell types (54). Of note, several studies have reported physical and functional interactions between Crk/CrkL proteins and the PI3K signaling pathway (55–57), thus providing a plausible framework for the mechanistic basis of the observed enhancement of NS1-mediated PI3K activation via the NS1-Crk/CrkL interaction. However, additional roles for the NS1-Crk/CrkL complex in cell biology of influenza A virus should obviously not be ruled out.

Previous studies concerning the activation of the PI3K pathway by NS1 have shown that this regulation involves an association of NS1 with the p85 regulatory subunit of PI3K (6, 8, 9), and subsequent studies even further suggested that there is a direct binding of NS1 to the SH3 domain of p85 (6). However, our studies failed to reveal measurable binding of the SH3 domains of p85α or p85β to NS1 proteins like A/Udorn and A/WSN, which contain the P*XX*P sequences pointed out by Shin *et al.* (6) or to A/Brevig or A/Mallard NS1 proteins, which contain the additional class II consensus SH3-binding motif described in this study. Our results do not exclude the possibility that weak binding of p85 SH3 to one of these nonconsensus P*XX*P motifs in NS1 might play a subtle role in stabilizing or coordinating NS1/p85 binding, but argue against a major role of such contacts in driving NS1-p85 complex formation.

It should be noted, however, that the ability of recombinant p85 SH3 to precipitate NS1 protein from lysates of influenza A-infected cells as reported by Shin *et al.* (6) does not prove this interaction to be direct rather than mediated by one or more additional factors. In fact, it has been reported that although association of NS1 with p85 can be readily detected in influenza A virus-infected cells, this is less true in the case of NS1 gene-transfected cells, leading Ehrhardt *et al.* (8) to propose that a cellular bridging factor is required for NS1-p85 complex formation. In this regard, it is interesting to note that although we found A/Udorn NS1 to be completely negative in the CrkL co-precipitation assay in transfected cells (Fig. 3), we did observe a weak association of NS1 and CrkL in cells infected with A/Udorn (data not shown). Thus, in influenza A virus-infected cells expressing high levels of NS1 (as well as other viral proteins that could also be involved) a large protein complex involving NS1, PI3K, and Crk/CrkL might form independently of direct binding of NS1 to Crk/CrkL or the p85 subunit of PI3K. However, NS1 proteins like those of A/Brevig and A/Mallard that can directly recruit Crk/CrkL via SH3 binding would serve to promote and strengthen the assembly of this multiprotein "signalosome" complex.

Enhanced PI3K Activation by NS1 via SH3 Binding

Further characterization of this NS1/PI3K/Crk-containing signalosome is clearly warranted and will shed new light into the cell biology of influenza A replication. Small molecular inhibitors of PI3K are already under evaluation as drugs in cancer (58, 59), and have been shown to reduce influenza A replication *in vitro* (7, 9). Thus, cellular signaling involving NS1/PI3K/Crk could also provide a useful target for the development of novel anti-influenza drugs. In this regard, possible involvement of the enhanced PI3K activation mediated via Crk/CrkL binding by NS1 proteins of avian strains of influenza in regulating interspecies transmission or pathogenicity in human hosts deserves special attention.

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Reorganization of the host cell Crk(L)–PI3 kinase signaling complex by the influenza A virus NS1 protein

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ABSTRACT

The non-structural protein-1 (NS1) of influenza A virus binds the p85β subunit of phosphoinositide 3-kinase (PI3K) to induce PI3K activity in the infected cells. Some virus strains encode NS1 containing a motif that binds tightly to the SH3 domain of the cellular adapter proteins Crk and CrkL to potentiate NS1-induced PI3K activation. Here we show that this potentiation involves reorganization of the natural CrkL–p85β complex into a novel trimeric complex where NS1 serves as a bridging factor. Of note, NS1 proteins that lack the SH3 binding capacity can also associate with CrkL, but in a less stable trimeric complex mediated by p85β. The data presented here establish Crk proteins as general host cell cofactors of NS1, and show that the enhanced PI3K activation by SH3 binding-competent NS1 variants is mediated by a more efficient tethering of Crk proteins to the NS1–PI3K complex.

Introduction

Influenza A virus belongs to the *Orthomyxoviridae* family of enveloped viruses (Palese and Shaw, 2007). Its genome is organized into eight single stranded negative-sense RNA segments and encodes up to 12 different proteins (Medina and Garcia-Sastre, 2011). The non-structural protein-1 (NS1) is a multifunctional virulence factor that is expressed at high levels in infected cells. The multiple protein binding motifs and conformational plasticity of NS1 enables it to interact with a plethora of host cell factors (Hale, 2014).

NS1 is the key innate immunity evasion factor of influenza viruses, and can hinder the interferon response via multiple mechanisms (Krug and Garcia-Sastre, 2013). In addition, NS1 contributes to activation of the phosphoinositide 3-kinase (PI3K) signaling in influenza A virus-infected cells. NS1 binds specifically to the regulatory PI3K subunit p85β (Hale et al., 2006; Shin et al., 2007) via an interaction that involves the NS1 residues Tyr89 and Pro164 contacting the linker region between the two Src-homology-2 (SH2) domains of p85β (Hale et al., 2010). Together biochemical and structural studies on this interaction have led to a model in which NS1 activates PI3K catalytic activity both by neutralizing a negative regulatory element in the inter-SH2 region of p85β as well as via direct contacts with the catalytic PI3K subunit p110 (Hale et al., 2008, 2010).

Activation of PI3K signaling is not specific for IAV infection, and instead is a common theme observed in host cell interactions of a variety of RNA and DNA viruses. Increased PI3K activity can benefit viral replication via several mechanisms, including promotion of viral entry, enhanced viral replication and gene expression, and blocking of premature apoptosis of the host cell (Buchkovich et al., 2008; Cooray, 2004; Dunn and Connor, 2012; Ehrhardt and Ludwig, 2009), but has also been shown to be involved in induction of antiviral innate immunity (Hrincius et al., 2011). Nevertheless, the net effect of PI3K activity on IAV replication is typically positive, as demonstrated by reports on profound suppression of viral replication observed upon treatment of infected cultures with PI3K inhibitors (Ehrhardt et al., 2006; Hale et al., 2006).

While NS1 proteins from most if not all influenza A viruses are capable of inducing at least some level of PI3K activation, our studies have revealed that the 1918 pandemic influenza virus and many avian IAVs are exceptionally potent PI3K activators (Heikkinen et al., 2008). This distinct capacity was found to be due to the presence of a functional SH3 domain-binding site close to the C-termini of these NS1 proteins, which is lacking from NS1 proteins of influenza A strains causing seasonal epidemics in humans.

Although the p85β protein also contains an SH3 domain, we could not observe any binding of NS1 to this SH3 domain (Heikkinen et al., 2008). Instead, we found that the NS1 SH3-binding motif mediated strong and selective binding to the

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aminoterminal SH3 domains of the two related adapter proteins, Crk and CrkL (Heikkinen et al., 2008). In the following we use the term Crk(L) for all Crk-family proteins, and CrkI, CrkII, or CrkL only when specifically referring to one of them. SH3-dependent binding of NS1 to Crk(L) has subsequently been confirmed by Ehrhardt and colleagues, who also reported on the role of this interaction in suppression of the JNK–ATF2 pathway (Hrincius et al., 2010). Moreover, a functional Crk(L) binding motif has also been linked to NS1-induced increased in cell viability in a complex also involving NS1-binding protein-1 (Miyazaki et al., 2013), as well as in dissociation of cellular Crk–Abl protein complexes and in supression of the tyrosine kinase activity of Abl (Hrincius et al., 2014).

Crk proteins are involved in PI3K signaling by binding to a proline-rich motif in p85 via their N-terminal Crk(L) SH3 domain (Brehme et al., 2009; Gelkop et al., 2001; Sattler et al., 1996). This interaction has been studied especially regarding its role in PI3K regulation upon immune cell activation. Studies in T cells have shown that the interaction between Crk and p85 is coordinated and facilitated by the ubiquitin ligase Cbl, which can engage in additional interactions both with Crk and p85 (Gelkop et al., 2001). The Crk–p85–Cbl protein complex has also been characterized as an important co-factor in oncogenic signaling by the Bcr–Abl fusion protein in chronic myeloid leukemia (Sattler et al., 1996). Indeed, an extensive proteomic characterization of the molecular network involved in Bcr–Abl function by Superti-Furga and colleagues identified Crk, p85, and Cbl among the seven most highly interconnected core components of this signaling network (Brehme et al., 2009).

In order to elucidate the biochemical mechanism underlying the PI3K superactivation by the SH3 binding-competent NS1 proteins we now have characterized in detail the mutual interaction between NS1, p85β, and CrkL. Because both NS1 and p85β interact with the Crk proteins via the same N-terminal Crk(L) SH3 domain it was of specific interest to investigate whether a trimeric complex involving p85β, Crk(L) and NS1 can form.

Results

Association of different Crk proteins with NS1 and p85β

The family of Crk adapter proteins includes three members, CrkI, CrkII, and CrkL. Of these CrkII (40 kDa) and CrkI (28 kDa) are products of the same gene, whereas CrkL (39 kDa) is encoded by a different gene. CrkII and CrkL consist of one SH2 and two SH3 domains, while CrkI is truncated before the second SH3 domain due to alternative mRNA splicing (Fig. 1a).

To extend our previous findings on NS1/Crk(L) binding (Heikkinen et al., 2008), we compared the capacity of these three different Crk proteins to interact with a representative avian NS1 protein containing a functional SH3 binding site (NS1 from A/Mallard/Netherlands/12/2000/H7N3; “Mallard” in the following). To examine the role of NS1 SH3 binding, we also included in these experiments a mutated version of Mallard NS1 protein (NS1-AxxA), carrying an SH3 binding site disrupted by alanine substitution of two critical proline residues (P212 and P215). As shown in Fig. 1b, NS1 associated readily with CrkI, CrkII, as well as CrkL, correlating with the presence of an N-terminal SH3 domain in all of these Crk proteins (Fig. 1a). In contrast, none of the Crk proteins co-precipitated the SH3 binding-deficient NS1-AxxA. The blotting data shown in Fig. 1b are representative of several experiments, and all results shown have been reproduced at least three times. The same holds true for the blotting data shown in Figs. 2, 4, 5 and 6.

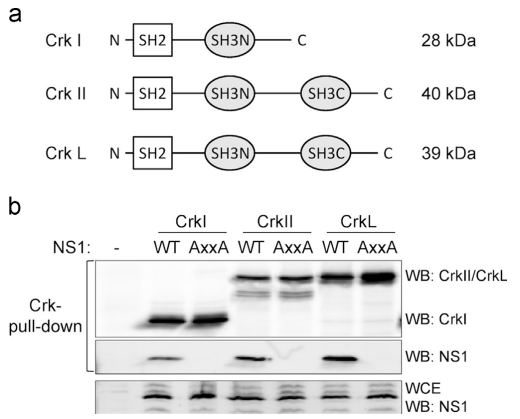

Fig. 1. All Crk proteins have a comparable and SH3 domain-dependent capacity to bind NS1. (a) Domain organization of CrkI, CrkII, and CrkL. (b) Co-precipitation of wild-type (WT) and SH3 binding deficient mutant (AxxA) Mallard NS1 proteins with CrkI, CrkII, and CrkL from 293T cells. Crk proteins fused with a biotinylation domain were cotransfected into cells with myc-tagged NS1 proteins. The amounts of NS1 in the transfected lysates (bottom panel) or co-precipitated with Crk proteins by streptavidin-coated beads (middle panel) were examined by anti-Myc Western blotting. Equal precipitation of the biotinylated Crk proteins was confirmed by blotting with labeled streptavidin (top panel).

Relative roles of p85β and Crk-SH3 binding in PI3K activation by NS1

Hale and colleagues reported the critical contribution of the tyrosine 89 of NS1 in direct binding to the regulatory PI3K subunit p85β (Hale et al., 2006). On the other hand, we have shown that NS1 containing a functional Crk-SH3 binding site show greatly enhanced PI3K activation compared to SH3 binding-deficient NS1 (Heikkinen et al., 2008). To investigate if the increased PI3K activation provided by NS1 SH3 binding was directly coupled to the NS1–p85β interaction or represented an independent regulatory event, we compared the effects of the AxxA (P212A/P215A; disrupting Crk SH3 binding) and Y89F (disrupting p85β binding) mutations.

In agreement with our earlier study (Heikkinen et al., 2008) we found that the PI3K-activating capacity of Mallard NS1 (induction of Akt phosphorylation) was reduced but not abrogated by the AxxA mutation (Fig. 2a). By contrast, the Y89F mutant NS1 was completely inactive in inducing Akt phosphorylation. Thus, we conclude that Crk(L) recruitment by NS1 indeed serves to enhance PI3K activation triggered by binding of NS1 to p85β, but this enhancement remains entirely dependent on the latter interaction.

Rearrangement of the endogenous p85β/CrkL complex by NS1

Because p85 binds not only to NS1 (Hale et al., 2006; Shin et al., 2007) but is also an established interaction partner of the Crk proteins (Brehme et al., 2009; Sattler et al., 1996, 1997) we examined the association of p85β with CrkL in untransfected cells and in cells transfected with the mutant NS1 proteins. Indeed, p85β could be readily co-precipitated with CrkL from untransfected cells that did not express any NS1 (Fig. 2b, leftmost lane). Transfection of the CrkL binding-competent (WT) or incompetent (AxxA) NS1 proteins did not significantly influence the net association of p85β with CrkL. However, transfection of the Y89F mutant of NS1 (which is CrkL binding-competent but p85β binding-deficient) potently inhibited p85β/CrkL association. Since

p85β and NS1 compete for the same N-terminal SH3 domain of CrkL, this observation suggested that NS1 could displace p85β from binding to CrkL due to a higher affinity for the CrkL SH3 domain.

To directly demonstrate that NS1 could displace p85β from a preformed p85β–CrkL complex, we co-transfected cells with CrkL and p85β without NS1, and added increasing amounts of recombinant NS1–Y89F into a lysate of these cells to examine whether p85β or NS1 co-precipitated with CrkL. As shown in Fig. 2c recombinant NS1–Y89F displaced p85β from its complex with CrkL in a dose-dependent manner. The highest dose (12 μg) of NS1–Y89F (corresponding to a 4-fold molar excess of p85β in the lysate) completely abrogated association of p85β with CrkL. By contrast, 12 μg of NS1-AxxA had no effect. As could be predicted, a similar displacement of 85β from the complex with CrkL by wild-type NS1 could be also demonstrated when NS1–p85β binding

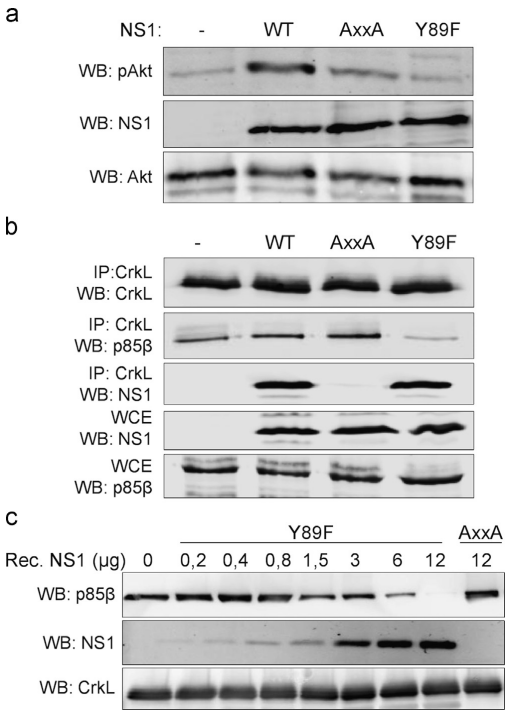

Fig. 2. Mutational analysis of the roles of SH3- and p85β-binding by NS1. (a) Activation of PI3K signaling by wild-type (WT), SH3-binding deficient (AxxA), or p85β-binding deficient (Y89F) NS1 proteins. Myc-tagged versions of the indicated NS1 proteins were transfected into Huh7 cells. 48 h later activation of PI3K signaling was assayed by Western blotting of lysates of the transfected cells with an antibody specific for phospho-Akt (top panel). Equal amounts of the different NS1 proteins in these lysates were confirmed by anti-Myc Western blotting (middle panel). (b) Co-precipitation of endogenous p85β and transfected NS1 proteins (WT, AxxA, or Y89F) with endogenous CrkL. Lysates of cells transfected as in A were subjected to anti-CrkL immunoprecipitation followed by Western blotting analysis of the immunocomplexes with an anti-p85β (middle panel) or an anti-Myc (NS1) antibody (bottom panel). Equal NS1 expression in the total lysates was confirmed as in (a). (c) Competitive disruption of the CrkL–p85β complex by titration of recombinant NS1–Y89F. Increasing amounts (from 0 μg to 12 μg) of recombinant NS1–Y89F or 12 μg of NS1-AxxA expressed as GST fusion proteins were added to lysates of cells transfected with biotinylation domain-tagged CrkL and HA-tagged p85β (total protein amount 200 μg). The amount of p85β and NS1 proteins associated with CrkL precipitated with streptavidin beads was examined by Western blotting.

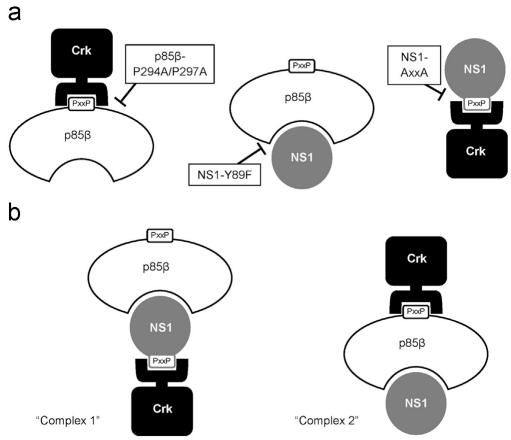

Fig. 3. Reported (a) and hypothesized (b) interactions among the Crk(L), p85β, and NS1 proteins. Note that all natural NS1 proteins do not contain the SH3 target motif (PxxP) formed by the NS1 residues P212 and P215. Hence this motif has been drawn only when it is necessary for the indicated complex. As discussed in the text, the affinity of the Crk(L) SH3 domain for the NS1 PxxP motif appears to be greater than its affinity for the p85β PxxP motif. The presence or absence of this motif in NS1 may therefore determine which of the two alternative trimeric complexes illustrated in (b) is formed. The references for the previously reported dimeric complexes shown in (a) are Crk(L)–p85β (Gelkop et al., 2001; Sattler et al., 1997), NS1–p85β (Hale et al., 2006; Shin et al., 2007), and in (b) NS1–Crk(L) (Heikkinen et al., 2008).

was prevented by specific mutations in p85β (p85β-V573M) (Supplementary Fig. 1).

These results suggested that co-precipitation of p85β with CrkL observed in cells expressing wild-type NS1 (Fig. 2b) was probably not direct, but instead indirectly mediated via the dual capacity of NS1 to bind both CrkL and p85β, thus indicating a scenario where the NS1-mediated enhancement of PI3K activation would involve a rearrangement of the natural CrkL–p85β complex and lead to formation of a trimeric CrkL–NS1–p85β complex (where NS1 is the bridging factor; see Fig. 3). Assembly of such a trimeric complex (as opposed to separate CrkL–NS1 and NS1–p85β complexes) is enabled by the abundant cellular expression of CrkL.

On the other hand, based on the capacity of Crk(L), p85, and NS1 to form dimeric complexes (see Fig. 3a), one could hypothesize that the complex of SH3 binding-deficient NS1 with p85β should also contain CrkL, thus representing an alternative trimeric CrkL–p85β–NS1 complex (where p85β is the bridging factor).

Seemingly in conflict with this assumption, only the NS1 proteins capable of direct CrkL binding (WT and Y89F) but no NS1-AxxA were found in anti-CrkL immunocomplexes (Fig. 2b). Since this could be explained by a modest affinity of the CrkL SH3 domain for p85β and/or a low abundance of p85β compared to CrkL and NS1, we examined if overexpression of p85β would allow us to detect p85β-mediated bridging of CrkL and NS1-AxxA. Thus, we co-transfected increasing amounts of p85β into cells together with a constant amount of wild-type NS1 or NS1-AxxA. As expected, coprecipitation of wild-type NS1 with CrkL was already efficient in the absence of p85β transfection, and did not significantly change upon ectopic p85β expression (Fig. 4a). By contrast, increasing amounts of p85β enabled coprecipitation of NS1-AxxA with CrkL in a dose-dependent manner, resulting in CrkL-association almost as efficient as observed for wild-type NS1. Thus, we concluded that a p85β-mediated CrkL–p85β–NS1 complex does form in cells expressing an SH3 binding-incompetent NS1 protein.

Demonstration of a p85β-bridged association of CrkL and the Mallard NS1-AxxA suggested that similar trimeric Crk(L)–p85β–NS1 complexes would also be formed in cells expressing NS1 proteins encoded by prototypic viruses, such as A/WSN/33 and A/Udorn/72, which represent seasonal human IAV strains lacking the Crk(L) SH3 binding motif. Indeed, when WSN and Udorn NS1 proteins were co-transfected with p85β, they could be co-precipitated with cellular CrkL as efficiently as the Mallard NS1 protein (Fig. 4b). In strong support of the model of the two alternative CrkL–p85β–NS1 complexes (Fig. 3b), the capacity of p85β to couple Udorn and WSN NS1 with CrkL completely depended on the SH3 binding motif of p85β, and was disrupted by the P294A/P297A mutation. Thus, we conclude that Crk proteins are also associated with the PI3K-activating NS1/p85β complexes involving NS1 proteins that lack direct Crk(L) binding capacity.

To further test the model of two alternative trimeric complexes (Fig. 3b), we co-expressed all relevant combinations of the differentially mutated versions of these proteins, and investigated in parallel which complexes could form (Fig. 5). For these studies we generated an additional mutant of p85β (V573M), which prevents the binding of p85β to NS1 (Li et al., 2008). As already shown for endogenous p85β (Fig. 2b), transfected wild-type p85β-HA could be co-precipitated with CrkL from wild-type NS1- and NS1-AxxA-transfected cells (Fig. 5, panel II, lanes 3 and 4), as well as in the absence of any NS1 (lane 2), whereas expression of NS1–Y89F inhibited this association (lane 5). In the absence of NS1 the V573M mutation in p85β did not affect binding to CrkL (lane 10), but this mutation rendered p85β–CrkL association sensitive to inhibition also by wild-type NS1 (lane 11). In keeping with the earlier work (Gelkop et al., 2001; Sattler et al., 1997), disruption of the SH3 binding site in p85β (P294A, P297A) abolished direct binding of p85β to CrkL (lane 6). Attesting to the role of NS1 as a bridging factor between CrkL and p85β in Complex 1 (Fig. 3b) co-precipitation of p85β-P294A, P297A with CrkL could be rescued by wild-type NS1 (Fig. 5, lane 7), but not by NS1-AxxA or NS1-Y89F (lanes 8 and 9). The same was true for co-precipitation of CrkL with p85β (panel VI). Only wild-type NS1 could mediate pull-down of CrkL by p85β–P294A, P297A, whereas co-precipitation of CrkL by wild-type p85β was observed even in the absence of any NS1. Of note, however, wild-type NS1 further increased CrkL/p85β-association, attesting to the efficient formation of a trimeric CrkL–NS1–p85β complex. This effect also evident in the reciprocal co-IP (panel II), albeit less pronounced presumably because of the higher relative abundance of transfected p85β compared to endogenous CrkL.

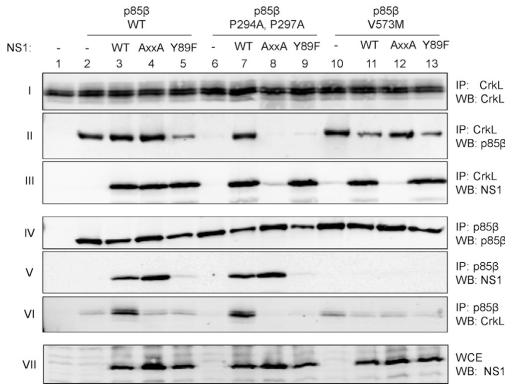

Fig. 5. Mutational characterization of the interaction interfaces involved in the assembly of two alternative trimeric complexes between CrkL, NS1, and p85β. Different combinations of wild-type and mutated versions of Mallard NS1 and p85β were transfected into 293T cells as indicated. Immunocomplexes precipitated from these cells by antibodies against CrkL (endogenous) (panels I-III) or p85β (anti-HA; panels IV-VI) were examined by probing the Western blot with antibodies against p85β (anti-HA), NS1, or CrkL as indicated. Expression of NS1 in all NS1-transfected cells was confirmed by Western blot analysis of total cell lysates (WCE).

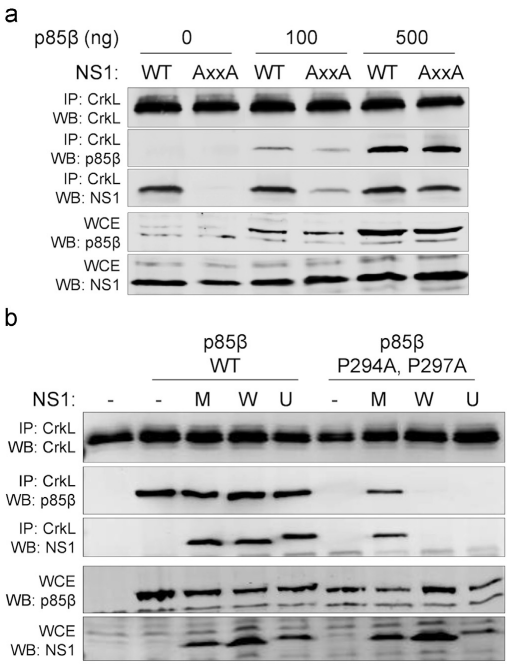

Fig. 4. Overexpression of p85β reveals association of CrkL with NS1/p85β complexes involving SH3 binding-incompetent NS1 proteins. (a) Increasing amounts of HA-tagged p85β was transfected to 293T cells together with wild-type (WT) or SH3-binding deficient (AxxA) NS1. Proteins associated with anti-CrkL (endogenous) precipitations from these cells were detected in Western blots with anti-HA (p85β), anti-CrkL, or anti-NS1 antibodies. The levels of transfected p85β and NS1 in the total lysates (WCE) were similarly detected. (b) NS1 proteins from Mallard (M), WSN (W), or Udorn (U) strains were cotransfected to 293T cells together with a wild-type (WT) or Crk(L) SH3 domain binding-deficient mutant (P294A, P297A) versions of p85β. The association of these NS1 with anti-CrkL immunocomplexes was examined as in (a).

In addition to the expected coprecipitation pattern of the p85β variants, the presence of NS1 and its AxxA and Y89F mutants in the anti-CrkL (panel III) or in the anti-p85β (anti-HA; panel V) immunocomplexes correlated perfectly with the model presented in Fig. 3(b). Specifically, V573M mutation in p85β or Y89F mutation in NS1 prevented interaction of NS1 with p85β, while SH3 binding capacity of NS1 did not play a role. In contrast, whereas the SH3 binding-competent NS1-WT and NS1–Y89F proteins could be precipitated with CrkL regardless of the cotransfected p85β variant, NS1-AxxA failed to associate with CrkL if either the SH3 binding site (P294A, P297A) or the NS1 binding site (V573M) of p85β was mutated.

To confirm that such trimolecular complexes can assemble as depicted in Fig. 3b without the assistance of additional CrkL- or p85β-associated cellular factors, we expressed and purified full-length p85β (both wild-type and the P294A/P297A mutant) and NS1 (both wild-type and the AxxA mutant) proteins, as well as a CrkL protein fragment containing the SH3 domain in recombinant

form. Experiments involving mixing of two or three of these proteins together followed by pull-down analysis of proteins associated with wild-type or mutant p85β confirmed that Complex I (where NS1 connects p85β and CrkL) as well as Complex II (where p85β connects NS1 and CrkL) can be assembled in vitro from purified recombinant components (Supplementary Fig. 2). Moreover, confirming our conclusions on the assembly of these complexes in living cells, SH3 binding-deficient NS1 could associate with CrkL in Complex II only if the SH3 binding site in p85β was intact.

In conclusion, these data firmly established a model where a trimeric complex involving NS1, CrkL, and p85β can assemble according to two distinct principles, depending on the SH3 binding capacity of NS1 (Fig. 3b).

Functional consequences of p85β/CrkL complex rearrangement by NS1

As already discussed, Crk proteins are naturally associated with PI3K via SH3-mediated binding to a proline-rich motif in p85β. Because of this interaction recruitment of CrkL to NS1–p85β complexes involving SH3 binding defective NS1 proteins was also observed (Figs. 4 and 5). However, we could detect a p85β-bridged CrkL–NS1 association (Complex 2) only when p85β was overexpressed, suggesting that CrkL recruitment to PI3K–NS1 complexes via p85β is less efficient than recruitment via an NS1–CrkL SH3 interaction. Thus, we reasoned that if the lower PI3K activation by SH3 binding-deficient NS1 proteins might be rescued by CrkL overexpression.

To test this idea we transfected wild-type Mallard NS1 and NS1-AxxA with or without CrkL, and compared the activation of PI3K in these cells (Fig. 6). Overexpression of CrkL had a relatively modest enhancing effect on wild-type NS1-induced Akt phosphorylation, whereas the weaker PI3K activation by NS1-AxxA was significantly potentiated by CrkL overexpression. When the naturally Crk(L) binding-deficient NS1 protein from the Udorn strain was tested, a similar potentiation of PI3K activation by CrkL overexpression could also be observed. Moreover, we also tested the capacity of overexpressed CrkI and CrkII to potentiate PI3K/Akt activation by Crk(L) binding-deficient NS1, and obtained results that were very similar to those seen with CrkL (data not shown).

Previously it was reported that the SH3 binding function of NS1 is involved in regulation of the c-Abl activity, a tyrosine kinase that phosphorylates tyrosine residues Y221 and Y207 in Crk and CrkL, respectively (Hrincius et al., 2014). Moreover, c-Abl could also phosphorylate p85β (Sattler et al., 1996). While an effective inhibition of c-Abl activity was evident by a profound suppression of CrkL tyrosine phosphorylation, we observed no effect on NS1-induced Akt phosphorylation (Supplementary Fig. 3), thus ruling out a critical role for c-Abl in this regulation.

In summary, we conclude that the efficient recruitment of Crk proteins into the PI3K–NS1 complex underlies the enhanced PI3K activation by SH3 binding competent NS1 proteins. However, additional qualitative differences between the two alternative Crk(L)–NS1–p85β might also exist and could favor PI3K activation in the context of Complex 1.

Discussion

Binding to the p85β regulatory subunit of PI3K is a conserved function of NS1 proteins of diverse IAV strains (Ehrhardt and Ludwig, 2009; Hale and Randall, 2007). This interaction leads to activation of PI3K/Akt signaling, which can contribute to viral replication and disease pathogenesis (see later). The capacity of NS1 of certain IAV strains to bind to the SH3 domains of the Crk

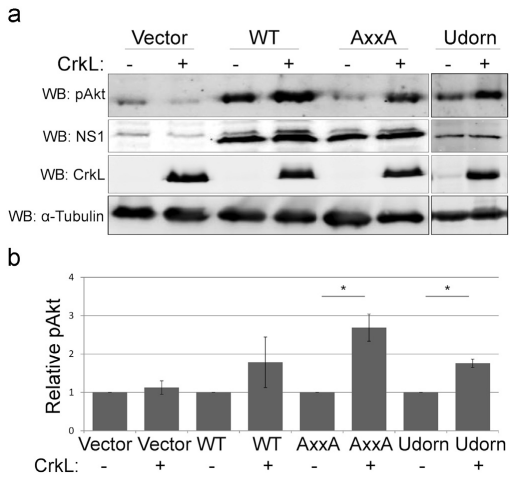

Fig. 6. Effect of CrkL overexpression on PI3K/Akt activation induced by various NS1 proteins. (a) Wild-type Mallard NS1 (WT), NS1-AxxA (AxxA), Udorn NS1 (Udorn) or an empty control vector (vector) were transfected into Huh7 cells with or without an expression vector for biotinylation domain-tagged CrkL. Induced PI3K/Akt signaling in the transfected cells was examined by probing the lysates with an antibody specific for phospho-Akt (top panel). The presence of NS1 and CrkL proteins in these lysates was detected with anti-Myc antibody or labeled streptavidin. The uniform loading of the lysates was controlled by probing with an anti-α-tubulin antibody (bottom panel). (b) Phospho-Akt signal intensities from (a) and two similar experiments were quantified using the Odyssey scanning software, and the enhanced pAkt signal intensity is shown as fold increase induced by CrkL overexpression as indicated. Statistical significance of the differences were determined by Student's *t*-test (*$P < 0.01$).

adapter proteins is associated with enhanced PI3K activation (Heikkinen et al., 2008). The same SH3 domain normally couples Crk proteins to PI3K by binding to a proline-rich target motif in p85pre (Gelkop et al., 2001; Sattler et al., 1997).

In this study we show that SH3 binding-competent NS1 proteins can displace this natural SH3 interaction, and direct the assembly of a novel Crk(L)–NS1–p85β complex ("Complex 1") where NS1 is the bridging factor. The interaction of p85β with NS1 lacking the capacity for SH3 binding also takes place in association with Crk(L), but involves an alternative trimeric complex NS1–p85β–Crk(L) ("Complex 2") where p85β is the bridging factor. Because of the low cellular levels of p85β, and the relatively modest affinity of p85β for the CrkL SH3 domain, Complex 2 could be physically observed only when p85β was overexpressed. Indeed, our data suggest that the enhanced PI3K activation in the context of Complex 1 is caused by a more efficient tethering of Crk(L) to NS1 and PI3K. In support of this conclusion, overexpression of CrkL could compensate for this difference, and thereby potentiate PI3K activation by SH3 binding-deficient NS1.

These results establish that enhancement of NS1-induced PI3K/Akt signaling by Crk(L) is directly coupled to the PI3K-activating NS1–p85β interaction, rather than acting somewhere upstream or downstream in the PI3K/Akt signaling pathway. Previous studies have found both Crk(L) and p85β in anti-NS1 immunocomplexes (Heikkinen et al., 2008; Hrincius et al., 2010), but this could also be explained by distinct dimeric NS1–p85β and NS1–Crk(L) complexes. In contrast, the combination of our mutagenesis and co-precipitation approaches provides a definite proof for mutual complexes involving all three proteins, and correlates the assembly of this trimeric complex with enhancemement of PI3K/Akt activation.

Importantly, our results also reveal a general role for Crk proteins as host cell cofactors in NS1-mediated activation of PI3K/Akt signaling, which is not limited to NS1 variants carrying a functional Crk(L) SH3 binding motif. However, the latter have clearly evolved a more efficient strategy to usurp Crk proteins as cofactors in PI3K regulation. The mechanistic role of Crk(L) in NS1-mediated PI3K/Akt activation remains to be fully understood. It is possible that Crk(L) or an additional Crk(L)-associated protein would be directly involved in the process where binding of NS1 leads to dismantling of the negative regulation imposed by p85β on the p110 subunit. Considering the role of Crk(L) as an archetypic cellular adapter protein, as well as the flexibility of the observed Crk(L)–NS1–p85β complex architecture, however, a perhaps more likely scenario is that Crk(L) serves as an anchor to localize the NS1–PI3K complex to a subcellular compartment that is favorable for activation of the PI3K/Akt pathway by NS1. Indeed, PI3K signaling is complex, involving several alternative p85 and p110 isoforms that can associate in many different combinations (Vanhaesebroeck et al., 2010). It is likely that these different types of PI3K variants operate in overlapping but different signaling pathways, and associate with distinct sets of partner proteins that regulate and target their activities. Thus, it is easy to envision how Crk(L) could be involved in guiding the NS1–PI3K complex to a specific cellular signaling domain that optimally supports PI3K activation as well as otherwise ensures a cellular response that maximally benefits the virus.

The NS1 gene of most seasonal IAV strains would need just a single nucleotide change to acquire the capacity to encode a functional Crk(L) SH3 binding motif, which therefore would be expected happen quickly if it provided direct replicative advantage for the virus. Thus, the reason why viruses like the 1918 pandemic strain have incorporated this feature into their genome must reflect part of a more complex strategy that they have evolved to interact with PI3K-dependent pro- and antiviral processes in their hosts.

Regulation of host cell PI3K activity during different steps of the IAV life-cycle is a multifaceted process that is not limited to the action of NS1 (see Ehrhardt and Ludwig, 2009). The role of PI3K activation by NS1 during IAV replication in cell culture and pathogenesis in mice has been studied using engineered viruses with mutant NS1 proteins that cannot bind to p85β. Originally it was reported that such mutants are attenuated, giving rise to a smaller plaque size and lower viral titers (Hale et al., 2006; Shin et al., 2007). More recently a profound reduction in viral replication and pathogenesis in mouse models of IAV infection was also reported (Ayllon et al., 2012b; Hrincius et al., 2012). However, the study by Hale and colleagues showed that the requirement for NS1-induced PI3K activation is strikingly virus strain dependent. While the underlying biology remains to be clarified (see Ayllon et al., 2012a), it is evident that variation elsewhere in the viral genome can render PI3K activation by NS1 largely or completely dispensable for IAV replication. On the other hand, only some (typically avian) strains of IAV encode SH3-binding competent NS1 proteins that can efficiently recruit Crk proteins to enhance NS1-induced PI3K activation (Heikkinen et al., 2008). As shown in the current study, however, Crk(L)-mediated potentiation of PI3K/Akt signaling depends entirely on p85β-binding coordinated by the tyrosine residue 89 of NS1. Thus, the variable capacity for Crk (L) SH3 binding by NS1 proteins from different IAV provides another layer of complexity to the strain-specific variation in host cell PI3K regulation.

In summary, the data presented in this study provide a detailed characterization of the Crk(L)–NS1–PI3K multiprotein complex, and emphasize the role of Crk proteins as host cell cofactors of the IAV virulence factor NS1. The fact that some IAV have incorporated a Crk(L) SH3 binding motif in the NS1 protein to take maximal use of the Crk proteins in order to enhance NS1-mediated PI3K activation attests the importance of PI3K-regulated host cell processes for IAV.

Materials and methods

Cells

Human embryonic kidney 293T (ATCC: CRL-11268) and human hepatocellular carcinoma Huh-7 (a gift from Mark Harris, University of Leeds, UK) cell lines were maintained in Dulbecco's modified Eagle's medium high glucose supplemented with 0.6 μg/ml penicillin, 60 μg/ml streptomycin, 10% fetal bovine serum, and 2 mM glutamine.

Plasmids and recombinant proteins

Plasmid constructs for the myc-NS1 (A/Mallard/Netherlands/12/2000/H7N3, A/Udorn/72 and A/WSN/33) proteins, GST-NS1, MBP-NS1 and the C-terminally biotinylated Crk proteins have been described before (Heikkinen et al., 2008; Kesti et al., 2007). The cDNA for mouse p85β (Open Biosystems) was cloned into the pEBB-vector (Tanaka et al., 1995) with an N-terminal HA-tag or a biotin acceptor domain. Codon changes in NS1 and p85β genes were generated by standard overlap PCR mutagenesis. GST-NS1 and MBP-NS1 recombinant proteins were produced as previously described (Heikkinen et al., 2008).

Protein precipitation and detection

For immunoprecipitation and protein pull-down experiments, 293T cells were transfected by a standard calcium phosphate precipitation method. 48 h after transfection the cells were collected and lysed in 1% NP40 lysis buffer (150 mM NaCl; 50 mM Tris–HCl, pH 7.9; 1% NP40). Cell lysates were used for immunoprecipitation with an anti-CrkL or an anti-HA antibody coupled to Dynabeads protein G magnetic beads (Invitrogen). Alternately lysates were used for streptavidin pulldown with streptavidin-coated Dynabeads (Invitrogen). To examine the phosphorylation status of Akt, Huh7 cells in 6-well plates were transfected with 4 μg of plasmid DNA using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Cells were serum-starved for 12 h and 48 h after transfection followed by lysis in 1% NP40 buffer. In Supplementary Fig. 3 the cells were treated with 20 μM Imatinib for 12 h. Western blots were visualized with the Odyssey infrared imaging system (LI-COR Biosciences).

Antibodies and inhibitors

The following primary antibodies were used in this study: mouse anti-Myc (9E10, Santa Cruz Biotechnology), mouse anti-CrkL (clone 5–6, Millipore), mouse anti-HA (F-7, Santa Cruz Biotechnology), rabbit anti-phospho Akt(Ser473) (D9E, Cell Signaling Technology), mouse anti-Akt (40D4, Cell Signaling Technology), mouse anti-p85β (T15, AbD Serotec), mouse anti-α-tubulin (DM1A, Sigma-Aldrich) and guinea-pig anti-NS1 (Melen et al., 2007). Streptavidin IRDye680CW, Streptavidin IRDye800CW, IRDye680CW goat anti-mouse IgG, IRDye800CW goat anti-mouse IgG, IRDye680CW goat anti-rabbit IgG, and IRDye800CW goat anti-rabbit were from LI-COR Biotechnology. IRDye800CW rabbit anti-guinea pig was from Rockland Immunochemicals. The c-Abl inhibitor Imatinib was from Sigma-Aldrich.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2015.06.009.

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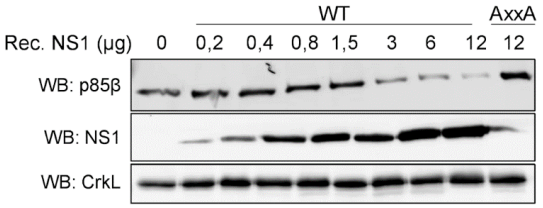
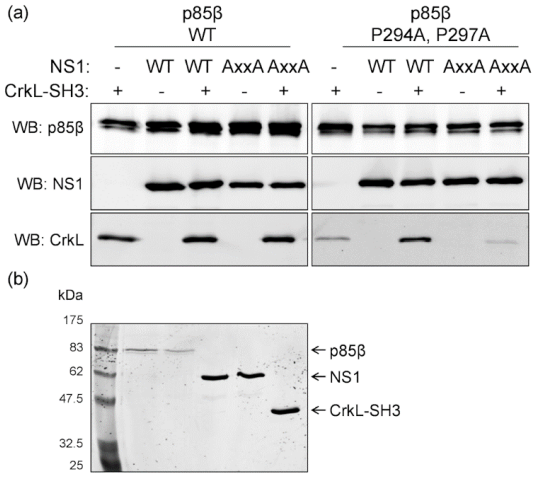


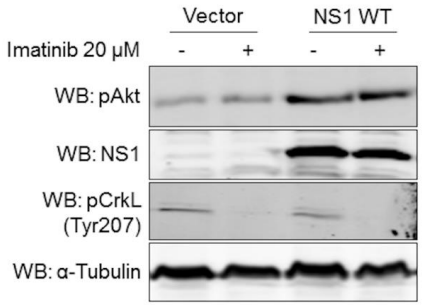
Supplementary Fig. 1.

Competitive disruption of the CrkL/p85β-V573M complex by titration of recombinant wild-type NS1. Increasing amounts (from 0 µg to 12 µg) of recombinant wild-type NS1 or 12 µg of NS1-AxxA expressed as GST fusion proteins were added to lysates of cells transfected with biotinylation domain-tagged CrkL and HA-tagged p85β-V573M (total protein amount 200 µg). The amount of p85β and NS1 proteins associated with CrkL precipitated with streptavidin beads was examined by Western blotting.



Supplementary Fig. 2.

Complex formation with purified proteins. (a) Biotinylation domain-tagged wild-type and P294A/P297A mutant p85β proteins (200 ng per reaction) purified from 293 cell cultures were mixed as indicated with MBP-tagged wild-type or AxxA mutant NS1 proteins (500 ng per reaction) and/or GST-tagged SH3 domain-containg fragment of CrkL (2 μg per reaction). NS1 and CrkL proteins co-precipitating with p85β proteins on streptavidin-coated particles were detected with an anti-NS1 antibody (middle panel) and anti-GST antibody (bottom panel). Despite some unavoidable background binding (evident also in the absence of any NS1) of the GST-tagged CrkL fragment to the p85β-P294A/P297A-containing beads, the specific capacity of wild-type NS1 (but not NS1-AxxA) to couple CrkL to p85β-P294A/P297A is clearly evident. (b) Quality and purity of the proteins used in (a) analyzed by SDS-PAGE and Coomassie staining.



Supplementary Fig. 3.

Effect of c-Abl inhibition on PI3K/Akt activation induced by NS1. Wild-type Mallard NS1 (WT) or an empty control vector (vector) was transfected into Huh7 cells. The cells were serum starved and treated or left untreated with 20 μM Imatinib for 12 h. Induction of PI3K/Akt signaling was examined by probing the lysates with an anti-phospho-Akt antibody (top panel), and expression of NS1 detected with an anti-Myc antibody (upper middle panel). To control the effect of Imatinib treatment, antibody specific for phospho-CrkL (Tyr207) was used (lower middle panel). Even loading of the gel was confirmed with an antibody for cellular α-tubulin (bottom panel).

III

viruses

MDPI

Article

Nuclear Translocation of Crk Adaptor Proteins by the Influenza A Virus NS1 Protein

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Abstract: The non-structural protein-1 (NS1) of many influenza A strains, especially those of avian origin, contains an SH3 ligand motif, which binds tightly to the cellular adaptor proteins Crk (Chicken tumor virus number 10 (CT10) regulator of kinase) and Crk-like adapter protein (CrkL). This interaction has been shown to potentiate NS1-induced activation of the phosphatidylinositol 3-kinase (PI3K), but additional effects on the host cell physiology may exist. Here we show that NS1 can induce an efficient translocation of Crk proteins from the cytoplasm into the nucleus, which results in an altered pattern of nuclear protein tyrosine phosphorylation. This was not observed using NS1 proteins deficient in SH3 binding or engineered to be exclusively cytoplasmic, indicating a physical role for NS1 as a carrier in the nuclear translocation of Crk. These data further emphasize the role of Crk proteins as host cell interaction partners of NS1, and highlight the potential for host cell manipulation gained by a viral protein simply via acquiring a short SH3 binding motif.

Keywords: NS1; influenza A virus; SH3 domain; Crk; virus-host interaction

1. Introduction

Influenza A virus (IAV) belongs to the *Orthomyxoviridae* family of enveloped viruses. It has a segmented genome consisting of eight single stranded negative-sense RNA strands. The non-structural protein 1 (NS1) of IAV is an important virulence factor, and a remarkably multifunctional protein that acts in several different ways to facilitate IAV replication (for reviews, see [1,2]).

The dynamic localization of NS1 in the nucleus as well as in the cytoplasm of IAV-infected cells is mediated by two nuclear localization signals (NLS) and by one nuclear export signal (NES) [3–5]. Soon after IAV infection, newly synthesized NS1 accumulates in the nucleus, but at late time points of infection it is transported into the cytoplasm. The conserved NLS1 of NS1 protein involves the amino acids R35, R37, R38, and K41 [3,6], while NLS2 is virus strain-specific, and it is located in the C-terminus of the protein [3,6,7]. The NES is located between the amino acids 138–147, leucine residues 144 and 146 being critical for its function [8,9].

The NS1 protein has several reported functions both in the nucleus and in the cytoplasm. In the nucleus, NS1 can inhibit cellular mRNA maturation and export by interacting with cleavage and polyadenylation specificity factor (CPSF), poly(A)-binding protein II (PABPII), mRNA splicing machinery, and nuclear export factors [10–12]. In the cytoplasm, NS1 prevents the activation of interferon-inducing proteins by inhibiting RNA helicase retinoic acid inducible gene-I (RIG-I) through a direct interaction [13,14], and by preventing RIG-I ubiquitination via interacting with ubiquitin E3

ligases TRIM-25 and Riplet, [15,16]. NS1 also inhibits the activity of protein kinase R (PKR) [17], and 2′-5′-oligoadenylate synthetase (OAS) [18], two important interferon-induced antiviral proteins.

In addition, NS1 can activate the host cell phosphatidylinositol 3-kinase (PI3K) cascade, a signaling pathway intimately involved in viral replication and innate immunity, by interacting directly with p85β, a regulatory subunit of the PI3K complex [19,20]. PI3K activation is further enhanced by NS1 proteins that contain an SH3 binding motif, which mediates a strong and selective binding to the cellular adaptor proteins Crk (Chicken tumor virus number 10 (CT10) regulator of kinase) and Crk-like adaptor protein (CrkL) [21]. This NS1 SH3 binding motif is commonly found in avian IAVs, but only in some human IAV strains, including the 1918 pandemic Spanish flu virus. This potentiation of PI3K activation involves reorganization of the cellular p85β-Crk protein complex. While SH3 binding-incompetent NS1 proteins simply bind to p85β in this complex, PI3K-superactivating NS1 proteins hijack the SH3 domain of Crk, thereby breaking the pre-existing p85β-Crk complex and assembling an alternative trimeric complex where NS1 is a bridging factor between p85β and Crk [22].

Crk proteins consist of a family of three members: CrkI, CrkII, and CrkL. CrkII and CrkL both contain one SH2 and two SH3 domains, while CrkI is a truncated form of CrkII that due to an alternative mRNA splicing possess only the SH2 and the N-terminal SH3 domain [23,24]. Although Crk proteins lack any enzymatic activity, they play a crucial role in cell biology by serving as essential adaptor proteins linking together different signaling molecules, such as tyrosine kinases and small G proteins through their SH2 and SH3 domains. They coordinate numerous biological processes, ranging from cell proliferation, cell adhesion and migration, phagocytic and endocytic pathways, apoptosis, and regulation of gene expression (for reviews, see [25,26]). The SH2 and SH3 domains of Crk proteins are highly homologous and display similar binding preferences and they have several overlapping roles, for example, in maintaining the cell structure and motility in mouse embryonic fibroblast (MEF) cells [27]. Use of knockout mice has revealed also some non-overlapping roles for these proteins in embryonic development. Knockout of CrkI/II or CrkL individually leads to different developmental defects in mice and they die perinatally [28,29]. Most of the cellular functions described for Crk proteins involve coordination of cytoplasmic signaling processes. However, Crk proteins have also been reported to enter the nucleus to regulate additional signaling pathways involved in malignant transformation and programmed cell death. The nuclear partners for Crk proteins are not well known, but prominently include the tyrosine kinase c-Abl, whose nuclear functions are important in cellular responses to DNA damage, cell cycle progression, and apoptosis [30]. Moreover, nuclear translocation of CrkII and its interaction with the nuclear tyrosine kinase Wee1 has been reported to be proapoptotic [31,32]. It has also been reported that the binding of CrkL to phosphorylated form of signal transducer and activator of transcription (STAT5) leads to translocation of the complex into the nucleus where it binds to the promoter region of *c-Abl* or *Bcr-Abl* genes in chronic myeloid leukemia (CML) cells [33,34]. Regulation of the nuclear entry of Crk proteins is not well understood. CrkII and CrkL have a nuclear export signal located in theC-terminal SH3-domain [35], but all Crk proteins lack a canonical nuclear localization signal, and apparently they can enter the nucleus only through interaction with other proteins that contain a functional NLS [36].

Since both NS1 and Crk have distinct nuclear and cytoplasmic functions, and since the effects on cellular physiology described for nuclear Crk proteins appear to depend on interaction partners that are actively transported into the nucleus, we examined how NS1 might influence the intracellular distribution of Crk proteins. Here we report that infection of cells with IAV encoding NS1 proteins that are competent for Crk binding, in contrast to viruses encoding NS1 lacking the SH3 ligand motif, cause a robust translocation of Crk proteins from the cytoplasm into the nucleus, which is associated with a noticeable change in tyrosine phosphorylation pattern of proteins in the nuclear fraction.

2. Materials and Methods

2.1. Cell Culture

The human lung epithelial (A549) and the human hepatocellular carcinoma (Huh-7) cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Sigma Aldrich, St. Louis, MO, USA) supplemented with 4500 mg/L of glucose, 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA), 0.05 mg/mL penicillin, 0.05 mg/mL streptomycin (Sigma Aldrich), and 1 mM L-glutamine (Sigma Aldrich) at 37 °C in 5% CO_2.

2.2. Recombinant Influenza A Viruses

The recombinant influenza A viruses were generated by using a plasmid-based reverse genetics as previously described [37]. A/WSN/1933 IAV was used as the background virus. The NS segment originated from either A/WSN/1933/H1N1 or A/Mallard/Netherlands/12/2000/H7N3 virus. The codon changes to NS1 sequence (A/WSN T215P; A/Mallard K217E) were introduced using overlapping polymerase chain reaction (PCR) mutagenesis. Influenza A/WSN/1933 recombinant viruses were propagated in 11-day-old embryonated chicken eggs at 34 °C for three days. The recombinant viruses used in this study are: A/WSN-NS1$^{Mallard(wt)}$, A/WSN-NS1$^{Mallard(K217E)}$, A/WSN-NS1$^{WSN(wt)}$, and A/WSN-NS1$^{WSN(T215P)}$.

2.3. DNA Transfections and Plasmids

A549 and Huh-7 cells were transfected by using a Lipofectamine 2000 reagent (Invitrogen, Waltham, Massachusetts, USA) according to manufacturer's instructions. The vector for A/Mallard myc-NS1 wild-type (WT) has been described before [21]. To generate fluorescent fusion proteins, mCherry was fused to the N-terminus of A/Mallard NS1, and enhanced green fluorescent protein (eGFP) to the N-terminus of CrkL. To generate a cytoplasmic A/Mallard NS1 (Cyto), the NES from MAPKK1 (LQKKLEELEL) was inserted between the mCherry and NS1 coding sequences. In addition, the NLS1 of NS1 protein was mutated (R38A, R41A) by standard overlap PCR mutagenesis. All plasmid constructs were verified correct by DNA sequencing.

2.4. Antibodies

The following primary antibodies were used in this study: mouse monoclonal anti-CrkL (clone 5–6, Millipore, Billerica, MA, USA), mouse monoclonal anti-Crk (clone 22, BD Transduction Laboratories, San Jose, CA, USA), rabbit monoclonal anti-phospho Akt (Ser473) (D9E, Cell Signaling Technology, Danver, MA, USA), mouse monoclonalanti-α-tubulin (DM1A, Sigma-Aldrich), rabbit polyclonal anti-Histone H3 (Cell Signaling Technology), monoclonal mouse anti-phosphotyrosine (PY20, Santa Cruz Biotechnology, Dallas, TX, USA), and guinea-pig polyclonal anti-NS1 [3]. The secondary antibodies for Western blotting were: IRDye680CW goat anti-mouse IgG, IRDye680CW goat anti-rabbit IgG, and IRDye800CW goat anti-rabbit, and IRDye800CW rabbit anti-guinea pig were from LI-COR Biotechnology (Lincoln, NE, USA). Secondary antibodies for immunofluorescence staining were: AlexaFluor 488 goat anti-guinea pig IgG (Abcam, Cambridge, UK), and AlexaFluor 546 goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA). Nuclei were stained with Hoechst.

2.5. Immunoprecipitation and Detection

For immunoprecipitation A549 cells were infected with recombinant IAVs for 24 h, and the cells were collected and lysed in 1% NP40 lysis buffer (150 mM NaCl; 50 mM Tris–HCl, pH 7.9; 1% NP40). Cell lysates were used for immunoprecipitation with an anti-CrkL antibody coupled to Dynabeads protein G magnetic beads (Invitrogen). To examine the phosphorylation status of Akt, Huh7 cells on 6-well plates were transfected with 4 μg of plasmid DNA. Transfected cells were serum-starved

for 12 h, and 48 h after transfection the cells were lysed in 1% NP40 lysis buffer. Western blots were visualized with the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA).

2.6. Cell Fractionation

A549 cells were seeded on 10 cm diameter well plates at 3 × 106 density. The next day, the cells were mock infected or infected with recombinant IAVs at a multiplicity of infections (MOI) 2 in the presence of 5 µg/mL of N-alpha-tosyl-L-phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma Aldrich). 24 h after infection the cells were scraped into 500 µL of ice cold Buffer A (20 mM Tris, pH 7.5, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2) supplemented with 0.5% Triton X-100. The cells were incubated on ice for 10 min and after that the nuclei were pelleted at 800 g for 10 min. The cytoplasmic extract (C) was collected and centrifuged at 16,100 g for 15 min. To prepare the nuclear extract (N), the nuclear pellet was washed once with Buffer A + 0.5% Triton X-100 and twice with Buffer A. The nuclei were suspended in 70 µl of Buffer B (20 mM·Tris, pH 8.0, 500 mM·NaCl, 2 mM·EDTA, pH 8.0, 0.1% Igepal) and sonicated for 3 s. The nuclear proteins were collected after centrifugation at 16,100 g for 15 min.

2.7. Immunofluorescence Staining and Confocal Imaging

For immunofluorescence microscopy, A549 cells were grown on coverslips and infected at an MOI of 0.5 in the presence of TPCK-treated trypsin (5 µg/mL). At 20 h after infection, the cells were fixed with ice cold methanol for 10 min at −20 °C, permeabilized with 0.1% Triton X-100, and incubated with guinea-pig anti-NS1 antibody, followed by AlexaFluor 488 goat anti-guinea pig IgG. CrkL was stained with mouse anti-CrkL antibody, followed by AlexaFluor 546 goat anti-mouse IgG. The cells were then examined with Leica TCS SP8 confocal microscope. Channels were scanned sequentially. The mean intensities of the CrkL fluorescence signal in the nuclei were analyzed by using the open source software, FiJi distribution of ImageJ (Version 1.50b, NIH) [38].

3. Results

3.1. SH3 Binding-Competent NS1 Proteins Translocate Crk Proteins into the Nucleus

To study the Crk/NS1 interaction in an infectious setting, we generated a set of recombinant viruses using a typical human IAV A/WSN/1933/H1N1 (A/WSN) as a background strain. These recombinant viruses are isogenic with wild-type A/WSN virus, except for the segment 8 (NS segment), which encodes either the wild-type or a mutated NS1 from an avian IAV A/Mallard/Netherlands/12/2000/H7N3 (A/Mallard) or a mutant construct of NS1 of A/WSN. To generate an SH3 binding-incompetent mutant of the A/Mallard NS1, a K217E mutation was introduced into its NS1 sequence. Conversely, to engineer the naturally SH3 binding-incompetent A/WSN NS1 to become SH3 binding-competent, a T215P mutation was introduced into its NS1 sequence. Although not directly relevant for this study, it should be noted that the T215P mutation could also alter the phosphorylation pattern of NS1 as T215 has been reported as a functional phosphorylation site [39,40]. The mutations made in the NS1 sequence do not affect the NS2/NEP open reading frame (ORF). The sequences of the relevant SH3-binding regions in the NS1 proteins of these viruses are shown in Figure 1A.

To establish that the engineered mutations had the expected effects on the capacity of the corresponding A/Mallard and A/WSN NS1 proteins to interact with Crk proteins in IAV infected cells, we immunoprecipitated endogenous CrkL (Figure 1B,C) from mock infected or recombinant virus-infected A549 cells and examined NS1 co-precipitation by Western blotting. As seen in Figure 1B, while wild-type A/Mallard NS1 readily co-precipitated with CrkL, the NS1 mutant (K217E) did not associate with CrkL at detectable levels. Conversely, no association of wild-type A/WSN NS1 with CrkL could be detected, whereas efficient co-precipitation of the mutant NS1-T215P protein with a restored Crk SH3-binding motif was observed (Figure 1C).

A

SH3-binding consensus	**P**xϕ**P**x**+**	Functional SH3-motif
A/Mallard WT	$_{212}$**P**P**LP**P**K**	+
A/Mallard K217E	$_{212}$**P**P**LP**P**E**	−
A/WSN WT	$_{212}$**P**P**L**T**PK**	−
A/WSN T215P	$_{212}$**P**P**LP**P**K**	+

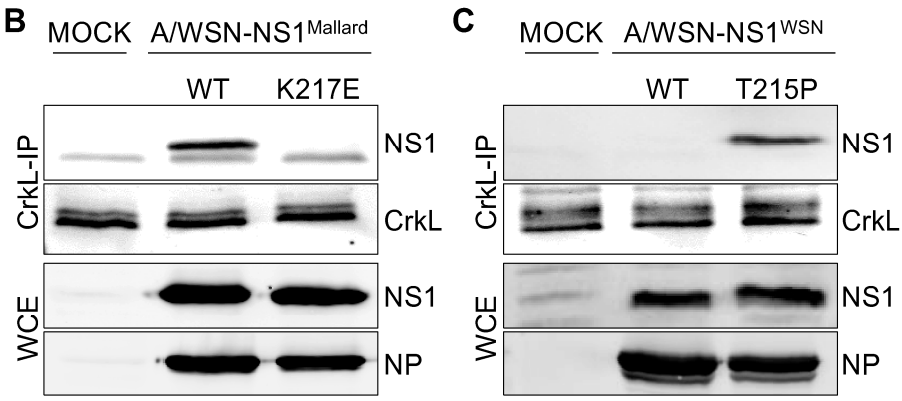


Figure 1. A functional SH3 binding motif in the non-structural protein-1 (NS1) is required for interaction with Crk-like adapter protein (CrkL) in influenza A virus (IAV)-infected cells. (**A**) The consensus sequence of class II SH3 binding motif, and its presence (**+**) or absence (−) in the C-terminal region (residues 212–217 shown) of NS1 proteins of the recombinant IAV strains used in this study. In SH3 binding consensus x indicates any residue, ϕ a hydrophopic residue, and + a positively charged amino acid, which for Crk-family SH3 domains is preferable a lysine residue; (**B,C**) Co-immunoprecipation of NS1 proteins with CrkL from lysates of A549 cells infected with recombinant A/WSN-based IAV strains expressing wild-type or mutant NS1 proteins derived from A/Mallard (**B**) or A/WSN (**C**) for 24 h at a multiplicity of infections (MOI) 2. Note that these NS1 proteins naturally differ in their SH3 binding capacity, and the mutations introduced in them thus have opposite effects. NS1 and nucleoprotein (**NP**) blots from whole cell extracts (**WCE**) before anti-CrkL immunoprecipitation are shown to control equal infection of the cells by the different viruses.

Next, we analyzed the localization of NS1 and Crk proteins in the infected cells by immunofluorescence staining and confocal imaging. A549 cells were infected with an MOI of 0.5 with A/WSN-NS1$^{Mallard(wt)}$ or A/WSN-NS1$^{Mallard(K217E)}$ recombinant viruses and the cells were fixed 20 h later. CrkL was localized mainly in the cytoplasm in mock-infected cells, and only faint staining was observed in the nucleus (Figure 2A, top row). In the infected cells, both the WT and the K217E-mutant NS1 proteins were predominantly localized in the nucleus (Figure 2A, in green). Strikingly, in cells infected with A/WSN-NS1$^{Mallard(wt)}$ CrkL was found to mainly co-localize with NS1 in the nucleus (Figure 2A, middle row), whereas in cells infected with A/WSN-NS1$^{Mallard(K217E)}$ the distribution of CrkL was indistinguishable from its predominantly cytoplasmic localization pattern in mock-infected cells (Figure 2A, bottom row). Very similar differential distribution was also observed for Crk when examined by immunostaining with an antibody that detects both CrkI and CrkII (data not shown). To more formally establish this effect, the mean intensity of CrkL-fluorescence signal in NS1-positive nuclei was quantified from 50 individual cells infected with A/WSN-NS1$^{Mallard(wt)}$, A/WSN-NS1$^{Mallard(K217E)}$, or mock-infected cells (Figure 2A, right panel). When the mean intensities

of CrkL immunostaining in these nuclei were normalized to the value of the mock-infected cells, a robust and highly significant nuclear translocation of CrkL by SH3 binding-competent but not by SH3 binding-incompetent NS1 could be demonstrated.

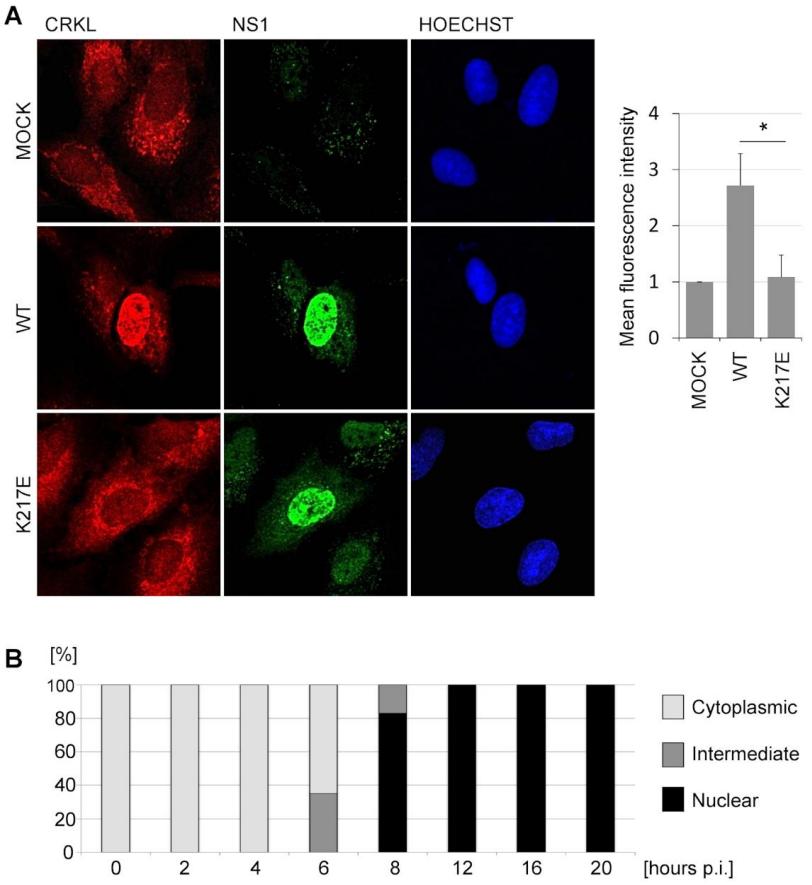


Figure 2. Infection of cells with IAV expressing SH3 binding-competent NS1 causes nuclear relocalization of CrkL. (**A**) Immunofluorescence staining of NS1 and CrkL in A549 cells that were mock-infected (upper panel) or infected with A/WSN-NS1$^{Mallard(wt)}$ (middle panel) or A/WSN-NS1$^{Mallard(K217E)}$ (bottom panel) for 20 h at a MOI 0.5. The nuclei were visualized by staining with Hoechst. The mean intensity of CrkL fluorescence in the nuclei was quantified from 50 cells infected with A/WSN-NS1$^{Mallard(wt)}$ or A/WSN-NS1$^{Mallard(K217E)}$ that also stained positive for positive for NS1, and was normalized to the mean fluorescence intensity of CrkL immunostaining of 50 mock-infected cells. The standard error is presented in the figure. The statistical significance of the differences was determined by Student's t-test (* $p < 0.001$); (**B**) A549 cells were infected with A/WSN-NS1$^{Mallard(wt)}$ for different time points at a MOI 0.5. The localization of CrkL was scored from 100 cells as a cytoplasmic, an intermediate, or a nuclear pattern.

When the kinetics of nuclear translocation of the Crk proteins was examined in more detail, we could observe first signs of nuclear accumulation of the Crk and CrkL at 6 h post-infection (p.i.) coinciding with the nuclei becoming clearly positive for NS1 staining (Figure 2B). At 8 h p.i. nuclear accumulation of Crk proteins was already prominent, and at 12 h p.i. Crk/CrL localization seemed already complete showing a pattern that looked identical to the 20 h p.i. time point shown in Figure 2A.

To extend and support these imaging studies by using a biochemical approach, we prepared cytoplasmic and nuclear fractions of cells infected with recombinant viruses and compared the presence of Crk proteins and wild-type or mutant NS1 proteins in these fractions (Figure 3A,B). The quality and purity of the nuclear and cytoplasmic protein fractions obtained from these cells were established by Western blotting of these preparations using antibodies against anti-α-tubulin (a cytoplasmic marker) and anti-histone H3 (a nuclear marker).

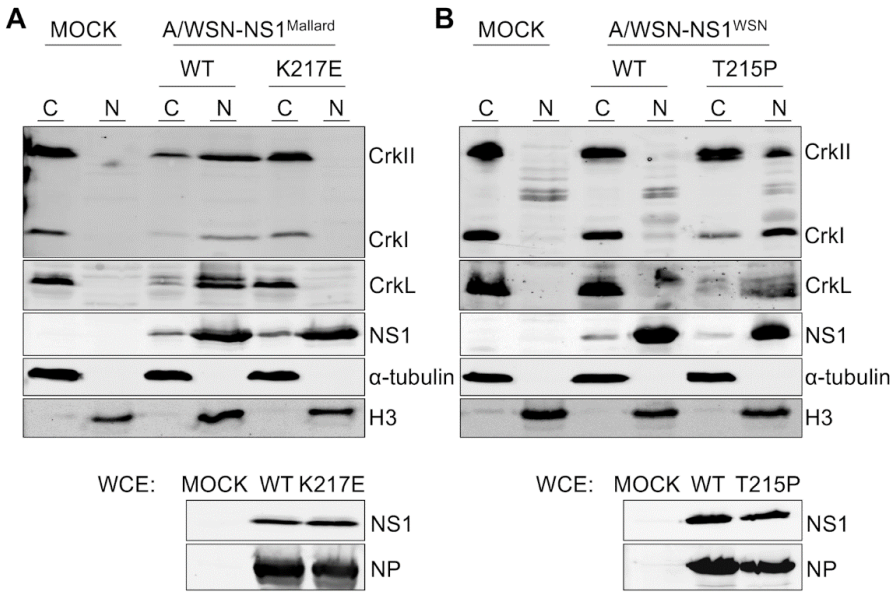


Figure 3. Nuclear translocation of CrkI, CrkII, and CrkL by SH3 binding-competent NS1 proteins demonstrated by subcellular fractionation. (**A**) Western blot analysis of cytoplasmic (**C**) and nuclear extracts (**N**) prepared from A549 cells that were mock-infected (**MOCK**) or infected for 24 h with recombinant A/WSN containing either the wild-type (**WT**) or the K217E mutant NS1 from A/Mallard virus at an MOI 2. In addition to antibodies against the Crk-family proteins and NS1, the blotted A549 fractions were also probed with antibodies against Histone H3 and α-tubulin to confirm successful separation of nuclear and cytoplasmic fractions. In addition, unfractionated whole cell extracts (**WCE**) of the infected cells were Western blotted with anti-NS1 and anti-NP antibodies to confirm uniform infection of cells by the different viruses; (**B**) Same as (**A**) except that the cells were infected with recombinant A/WSN virus carrying wild-type (**WT**) or the T215P mutant NS1 from A/WSN.

As expected, NS1 protein was seen mainly in the nuclear fractions regardless of the recombinant virus that was used to infect these cells. The nuclear fractions of mock-infected cells did not contain detectable amounts of CrkI, CrkII, or CrkL proteins (Figure 3A,B), whereas strong signals of expected size for these proteins were observed in the cytoplasmic fractions. Nuclear *vs.* cytoplasmic fractionation of Crk proteins derived from cells infected with viruses expressing an SH3 binding-incompetent version of NS1 (A/WSN-NS1$^{WSN(wt)}$ and A/WSN-NS1$^{Mallard(K217E)}$) was identical with that observed with mock-infected cells (Figure 3A,B). In sharp contrast, in cells infected with viruses having an SH3 binding-competent NS1 (A/WSN-NS1$^{WSN(T215P)}$ and A/WSN-NS1$^{Mallard(wt)}$) all Crk proteins could be abundantly detected in the nuclear fractions, especially CrkL becoming predominantly nuclear (Figure 3A,B).

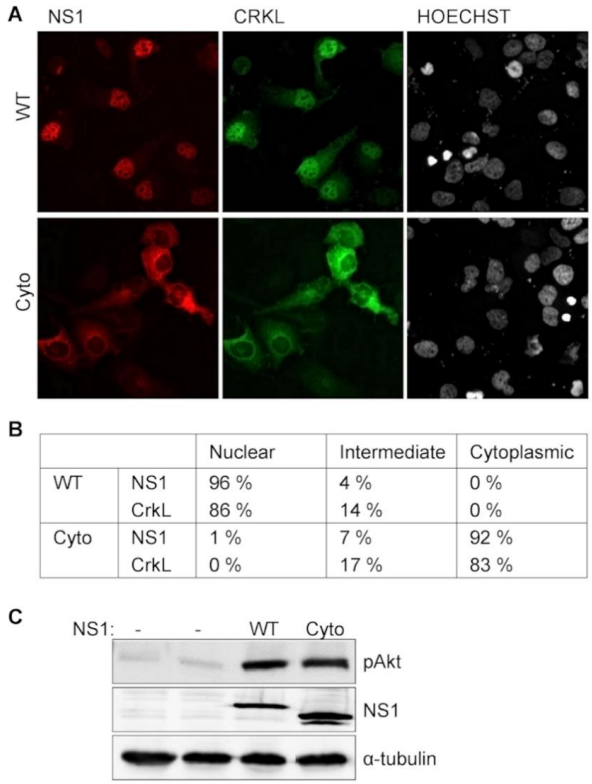
3.2. NS1-Induced PI3K-Activation does not Depend on Crk Relocalization into the Nucleus

Our previous studies have shown that simultaneous recruitment of Crk proteins by NS1 substantially potentiates NS1-induced activation of PI3-kinase pathway [21,22]. While these signaling interactions would be expected to take place in the cytoplasm, it is nevertheless possible that subsequent nuclear transit of the bulk of cellular Crk proteins by NS1 could somehow contribute to the observed PI3K superactivation.

To address this possibility we generated a mutant NS1 protein that remains predominantly in the cytoplasm (NS1-Cyto). This was achieved by mutating the N-terminal NLS1 of A/Mallard NS1 (this strain does not contain NLS2) at the critical basic residues (R38A,R41A) combined with the addition of a strong heterologous nuclear export signal (NES) from mitogen-activated protein kinase kinase-1 (MAPKK1) [41].

The localization of NS1-Cyto was compared with wild-type A/Mallard NS1 by transient transfection of red fluorescent fusion protein (mCherry) derivatives of these NS1 proteins. Similar to the NS1 immunostaining in IAV-infected cells, in virtually all productively NS1-transfected cells (96%; of 100 cells counted) the red fluorescence of wild-type NS1 showed a distinctly nuclear localization pattern as illustrated in Figure 4A (upper panel). By contrast, only 1% of cells transfected with NS1-Cyto showed such a nuclear fluorescence, and in almost all (92%) of these cells the nuclei were devoid of NS1 signal and appeared as dark areas inside cytoplasmic red fluorescence (Figure 4A, lower panel), thus establishing the success of our double mutation approach to generate an NS1 mutant restricted to a cytoplasmic localization. Co-transfection of a vector expressing CrkL tagged with eGFP recapitulated our results obtained by infection with recombinant IAV variants, showing a prominently nuclear green fluorescence that faithfully co-localized with wild-type NS1. Conversely, in NS1-Cyto-transfected cells also CrkL fluorescence was found predominantly in the cytoplasm (see Figure 4B for statistics of the observed NS1 and CrkL localization patterns). When eGFP-CrkL was transfected alone (data not shown), a localization pattern similar to that observed for endogenous CrkL by immunostaining (Figure 2A).

When the capacity of NS1-Cyto to trigger PI3K-activation in transfected cells was compared with that of wild-type A/Mallard NS1, a similar increase in the phosphorylation of Akt, a downstream effector of the PI3K cascade was observed (Figure 4C). Thus, while recruitment of Crk proteins by NS1 is required for the enhancement PI3K-activation [21,22], NS1-mediated nuclear translocation of Crk is not. Likewise, it can be concluded that while the cytoplasmic interaction between NS1 and Crk is sufficient to potentiate PI3K activation, nuclear targeting of Crk cannot be triggered by a contact with NS1 in the cytoplasm, but indeed physically depends on the nuclear entry of Crk-NS1-complex driven by the NLS of NS1.



		Nuclear	Intermediate	Cytoplasmic
WT	NS1	96 %	4 %	0 %
	CrkL	86 %	14 %	0 %
Cyto	NS1	1 %	7 %	92 %
	CrkL	0 %	17 %	83 %

Figure 4. NS1-mediated Crk relocalization is independent of NS1-activated PI3K-signaling. (**A**) Fluoresence microscopy imaging of Huh7 cells co-transfected with eGFP-fused CrkL (green fluorescense) together with mCherry-fusion protein (red fluorescence) of wild-type A/Mallard NS1 (WT) or its dominantly cytoplasmic mutant NS1-Cyto; (**B**) Localization of NS1 and CrkL was examined in 100 cells from (**A**) and the observed fluorescence patterns were scored as nuclear, intermediate, or cytoplasmic; (**C**) PI3K-activation by wild-type and mutant version of NS1 revealed by Akt phosphorylation. Huh7 cells were transiently transfected with a vector expressing the indicated NS1 variants, and 48 h later examined by Western blotting with antibodies against phospho-Akt (pAkt), NS1, and α-tubulin.

3.3. A Change in Nuclear Protein Tyrosine Phosphorylation after NS1-Mediated Nuclear Re-Localization of Crk

Crk proteins interact with many tyrosine phosphorylated proteins as well as tyrosine kinases [25], and upon the original discovery of the viral Crk oncogene (v-Crk) an increase in cellular protein tyrosine phosphorylation was described as a hallmark of Crk-mediated malignant transformation [42]. To study whether NS1-mediated nuclear translocation of Crk proteins in IAV-infected cells would lead to any functional consequences, we compared the patterns of protein tyrosine phosphorylation in nuclear extracts of A549 cells that were mock-infected or infected for 24 h with IAV expressing NS1 proteins either capable (A/WSN-NS1$^{\text{Mallard(wt)}}$ and A/WSN-NS1$^{\text{WSN(T215P)}}$) or not capable (A/WSN-NS1$^{\text{WSN(wt)}}$ and A/WSN-NS1$^{\text{Mallard(K217E)}}$) for binding and nuclear targeting of Crk. To enhance the accumulation of phosphotyrosine-modified proteins, the cells were treated for 10 min with the phosphotyrosine phosphatase-inhibitor pervanadate before they were fractionated into nuclear and cytoplasmic extracts that were subjected to Western blotting with an anti-phosphotyrosine (anti-pTyr) antibody (Figure 5). Successful subcellular fractionation was confirmed by probing with

antibodies against prototypic nuclear and cytoplasmic proteins, and uniform infection of the cells was demonstrated by probing unfractionated lysates of these cells with antibodies against IAV NS1 and NP. While the nuclear extracts of cells infected with viruses expressing NS1 proteins lacking Crk binding activity did not differ from mock-infected cells in their patterns of tyrosine phosphorylated proteins, a prominent new phosphotyrosine-containing protein with a MW of about 135 kDa appeared in the nuclear extracts of cells infected with A/WSN-NS1$^{Mallard(wt)}$ or A/WSN-NS1$^{WSN(T215P)}$ (Figure 5, pointed with arrows). Thus, we conclude that Crk proteins translocated into the nucleus upon IAV infection via their binding to NS1 can reprogram cellular signaling pathways in the nucleus as evidenced by altered nuclear protein tyrosine phosphorylation.

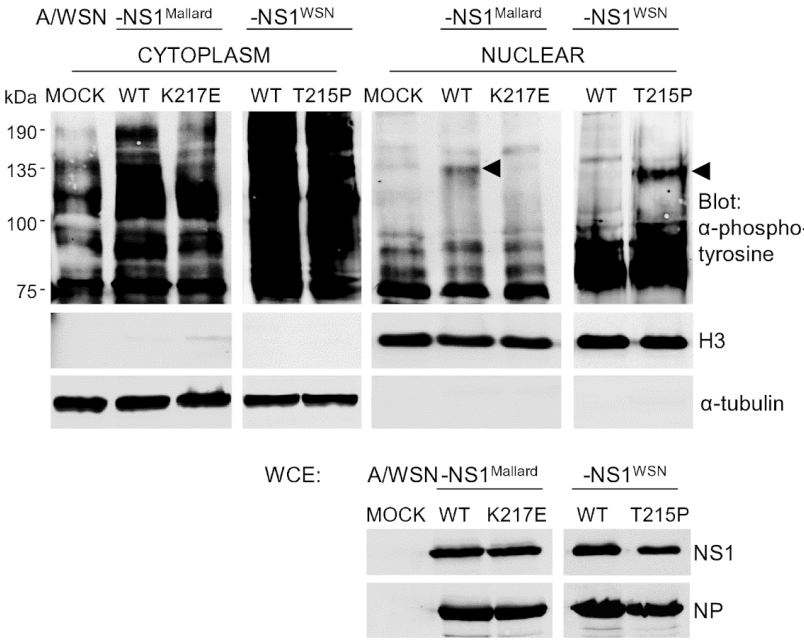


Figure 5. Nuclear targeting of Crk by NS1 causes a change in the nuclear protein tyrosine phosphorylation pattern of IAV-infected cells. A549 cells were infected with recombinant viruses as indicated for 24 h at a MOI 2, and treated with pervanadate for 10 min before cytoplasmic (**C**) and nuclear (**N**) extracts were prepared. The extracts were probed with an anti-phosphotyrosine antibody. As in Figure 3, shown are also H3 and α-tubulin blots to verify the quality of the subcellular fractionation, as well as blotting of whole cell extracts (WCE) with antibodies for NS1 and NP to verify equal NS1 expression of NS1 and uniform infection of cells with the different viruses.

4. Discussion

Acquiring a target motif for an SH3 domain-mediated interaction provides a convenient strategy for viruses to hijack key signaling pathways that regulate the behavior of their host cells. The high-affinity Crk SH3 binding site in the carboxyterminus of the IAV NS1 protein is an interesting example of the ease of such virus-host interaction evolution. As highlighted by the recombinant IAV strain A/WSN-NS1$^{WSN(T215P)}$ used in this study, a single nucleotide change in the segment 8 of the viral genome to change an ACT codon into CCT is sufficient to give rise to an NS1 protein with a capacity for fundamentally altering host cell physiology by taking the control of Crk-dependent signaling pathways. The degree of this control can be quite remarkable as evidenced by the dramatic relocalization of cellular Crk proteins to the nucleus described in this study.

Despite our present findings as well as other effects on the host cell previously assigned to the Crk SH3 interaction motif of NS1 [21,22,43,44], the overall role of SH3 binding capacity of NS1 in supporting IAV replication and pathogenesis remains unclear. Should this property alone provide a clear-cut replicative or immune evasion advantage, it would quickly become fixed in IAV evolution also in humans, which has not happened. Mutations analogous to our A/WSN-NS1$^{WSN(T215P)}$ mutant of the human IAV strains A/Udorn/72 and the 2009 Swine Flu pandemic virus (A/California/04/09) to introduce an SH3 binding site in NS1 did not result in enhanced viral replication [40,45]. On the other hand, another human IAV strain, A/PR8/8/34, was shown to benefit from the introduction of an SH3 binding motif to its NS1 and was more pathogenic in mice [46]. It is tantalizing to note that the 1918 pandemic Spanish flu virus A/Brevig Mission/1/18/H1N1 is one of the known human IAV strains that naturally contains this sequence motif, suggesting a positive contribution to viral fitness in this context [21]. It is likely that the utility of hijacking Crk signaling depends on a complex combination of other functional variables of IAV that are not only determined by the sequence variation in the multifunctional NS1 protein itself, but also encoded by other segments of its genome.

In addition to enhancing PI3K activation [21] via re-organization of the PI3K-Crk-complex [22], a functional Crk SH3 binding site of NS1 has previously been linked to suppression of c-Jun N-terminal kinase-activating transcriptional factor 2 (JNK-ATF2) pathway [43] and to an inhibition of the tyrosine kinase c-Abl [44]. Our current data suggest that this list may have to be extended to include many more of the diverse cellular functions of the Crk protein family. It should mentioned, however, that the interferon-antagonizing effects of NS1 do not fall into this category, and have been shown to be independent of Crk SH3 binding [21].

Nuclear relocalization of the bulk of cellular Crk proteins can be expected to affect several cytoplasmic Crk functions. However, phosphorylation and protein interactions are probably more relevant in regulation of these functions than the total cytoplasmic Crk concentration. This would explain why we did not observe the PI3K-NS1-Crk complex-dependent enhancement of PI3K activity in cells expressing an NS1 mutant that was forced to remain cytoplasmic and thus unable to move Crk into the nucleus. Perhaps more important than the reduction in the amount of cytoplasmic Crk may indeed be the triggering of new signaling events in the nucleus induced by NS1-mediated nuclear transportation of Crk.

Previous studies on cancer biology have described triggering of major signaling events and outcomes caused by nuclear transport of Crk proteins. CrkII has been reported to participate in apoptosis by activating caspases and binding to the nuclear cell cycle regulator, Wee1 through CrkII SH2 domain [31,32]. On the other hand, CrkL has been reported to bind via its SH2 domain to tyrosine phosphorylated Stat5 [34,47]. The complex can translocate into the nucleus to bind Stat5-responsive elements followed by regulation of gene expression [48,49]. It will be interesting to see how closely NS1-mediated nuclear relocalization of Crk recapitulates these events, and to what extent the complex with NS1 redirects Crk to alternative nuclear protein complexes and functions.

As a demonstration that NS1-mediated nuclear transport of Crk proteins can indeed reprogram nuclear signal transduction pathways, we showed the appearance of a novel nuclear tyrosine phosphorylated protein with an estimated molecular weight (MW) of 135 kDa (pp. 135). However, despite the established role of Crk proteins in regulating cellular pTyr protein levels, it should be noted that we cannot exclude the possibility that some SH3-dependent function of NS1 other than the observed robust nuclear transport of Crk could account for the associated changes in nuclear protein tyrosine phosphorylation.

The 135 kDa size of the novel pTyr-decorated nuclear protein matches with the major cytoplasmic tyrosine-phosphorylated protein partner of Crk proteins, the p130Cas [50,51]. However, while this remains a possible scenario, so far we have not been able to prove that cytoplasmic p130Cas is transported into the nucleus as a part of an NS1-Crk-p130Cas complex. Another candidate for pp. 135 that we have considered, but likewise not been able to prove is c-Abl, a partially nuclear [52] tyrosine kinase that can be activated by Crk [53] and undergo autophosphorylation [54]. However, since Crk

uses its N-terminal SH3 domain for binding to c-Abl [55], this interaction could take place only after dissociation of the NS1-Crk complex following its nuclear entry. At any case, further studies on the identity of pp135 as well as comprehensive analyses on the changes in the nuclear phosphoproteome induced by NS1-mediated nuclear transport of Crk proteins in IAV-infected cells clearly warrants further experimental attention in order to better characterize the functional significance of this novel function of NS1.

IAV is an unusual RNA virus in the sense that it replicates in the nucleus of the host cell. Thus, it is easy to understand why manipulation of the nuclear environment would be relevant for promoting the IAV life cycle. Ludwig and colleagues have reported that activation of the apoptotic effector caspase-3 at late stages of the IAV replication cycle is required for efficient nuclear exit of viral RNP complexes [56]. Given the previous reports on the capacity of nuclear Crk protein to promote apoptosis [31,32], a role of NS1-mediated nuclear transport of Crk in facilitating vRNP release from the nucleus poses one potentially interesting possibility. Since lamins are important caspase substrates and key components of the nuclear lamina, we have initiated studies on lamin cleavage and integrity of the nuclear lamina during IAV infection. Our preliminary data suggest that viruses expressing Crk binding-competent NS1 proteins could indeed induce lamin A/C cleavage and induce more extensive changes in the nuclear morphology than do viruses with NS1 proteins that lack the SH3 binding motif [57].

The present results further emphasize the role of Crk proteins as host cell interaction partners of IAV, although much work remains to be done to characterize the detailed nuclear functions of Crk protein relocalization to the nucleus by NS1, and the significance of this reprogrammed signaling for IAV replication and pathogenesis. Nevertheless, the remarkable potential of SH3 binding-competent NS1 proteins to robustly relocate a key family of host cell signaling factors from the cytoplasm to the nucleus attests to the extensive consequences that adopting a short protein interaction motif by a viral protein can have, and suggests that the role of the NS1-Crk interaction in cell biology of IAV may be broader than we have so far appreciated.

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